The mitogen-activated protein kinases (MAP kinases) play a central role in signaling pathways initiated by extracellular stimuli such as growth factors, cytokines, and various forms of environmental stress. Full activation of the MAP kinases requires dual phosphorylation of the Thr and Tyr residues in the TXY motif of the activation loop by MAP kinase kinases. Interestingly, down-regulation of MAP kinase activity can be initiated by multiple Ser/Thr phosphatases, Tyr-specific phosphatases, and dual-specificity phosphatases. This would inevitably lead to the formation of monophosphorylated MAP kinases. However, in much of the literature investigating MAP kinase signaling, there has been the implicit assumption that the monophosphorylated forms are inactive. Thus, the significance for the need of multiple phosphatases in regulating MAP kinase activity is not clear, and the biological functions of these monophosphorylated MAP kinases are currently unknown. We have prepared extracellular signal-regulated protein kinase 2 (ERK2) in all phosphorylated forms and kinetically characterized them using two proteins (the myelin basic protein and Elk-1) and ATP as substrates. Our results revealed that a single phosphorylation in the activation loop of ERK2 produces an intermediate activity state. Thus, the catalytic efficiencies of the monophosphorylated ERK2/pY and ERK2/pT (ERK2 phosphorylated on Tyr-185 and Thr-183, respectively) are 2–3 orders of magnitude higher than that of the unphosphorylated ERK2 and are only 1–2 orders of magnitude lower than that of the fully active bisphosphorylated ERK2/pTpY. This raises the possibility that the monophosphorylated ERK2s may have distinct biological roles in vivo. Different phosphorylation states in the activation loop could be linked to graded effects on a single ERK2 function. Alternatively, they could be linked to distinct ERK2 functions. Although less active than the bisphosphorylated species, the monophosphorylated ERK2s may differentially phosphorylate pathway components.

The mitogen-activated protein kinases (MAP kinases) play a central role in signaling pathways initiated by extracellular stimuli such as growth factors, cytokines, and various forms of environmental stress (1–3). MAP kinase cascades are conserved in organisms ranging from yeast to human. The three best-characterized MAP kinases are the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases, and the p38 kinases. A typical MAP kinase cascade consists of at least three kinases, a MAP kinase, a MAP kinase/ERK kinase (MEK) that activates the MAP kinase, and a MEK kinase (MEKK) that activates the MEK. Although ERKs respond robustly to growth factors and certain hormones, c-Jun N-terminal kinases and p38 kinases react primarily to cytokines and stress stimuli. After activation, each MAP kinase phosphorylates a distinct spectrum of substrates, which include key regulatory enzymes, cytoskeletal proteins, nuclear receptors, regulators of apoptosis, and many transcription factors. Such an array of substrates is consistent with the observation that MAP kinases are involved in many critical cell functions.

Like many protein kinases, the activity of MAP kinases is tightly regulated by phosphorylation. However, unlike many other protein kinases, whose activation requires phosphorylation of a single residue within a structurally conserved activation loop (4), the MAP kinases are activated by dual phosphorylation of the Thr and Tyr residues in the TXY motif of the activation loop (where X is Glu in ERKs, Pro in c-Jun N-terminal kinases, and Gly in p38 kinases) (5–7). Genetic and biochemical data suggest that the phosphorylation of both Thr and Tyr residues in each of the known MAP kinases is catalyzed by specific MEKs, which are dual specificity kinases (8–10). Because of its central role in signaling pathways regulating cell growth and differentiation, the prototypic member of the MAP kinase family ERK2 has been the subject of intense study. Biochemical studies have shown that phosphorylation at both sites in ERK2 is required for full kinase activity (5, 11, 12).

The kinase activity of the monophosphorylated ERK2/pT (ERK2 phosphorylated on Thr-183) and ERK2/pY (ERK2 phosphorylated on Tyr-185) has not been accurately measured. However, it is generally believed that the singly phosphorylated forms of ERK2 have little kinase activity. Monophosphorylated ERK2s can be produced by the action of MEK1, which phosphorylates ERK2 by a twocollision, distributive mechanism (13, 14). They can also be generated through the action of serine/threonine protein phosphatase PP2A (15) and tyrosinespecific PTP-SL (16) and HEPTP (17) on ERK2/pTpY (ERK2 phosphorylated on both Thr-183 and Tyr-185). Indeed, it has been shown that, in addition to the unphosphorylated ERK2 and the bisphosphorylated ERK2/pTpY, both ERK2/pT and ERK2/pY can be detected in living cells (18, 19). Although both acid; MTGuo, 2-amino-6-mercaptopurine (7-methyl-6-thioguanosine); PP, protein phosphatase; ERK2/pY and ERK2/pT, ERK2 phosphorylated on Tyr-185 and Thr-183; ERK2/pTpY, ERK2 phosphorylated on both Thr-183 and Tyr-185; PTP, protein tyrosine phosphatase; HEPTP, hematopoietic PTP.
of the monophosphorylated ERK2s exist in the cell, it is not clear whether they have any distinct biological functions in signaling. To begin to assess the potential biological significance of differential ERK2 phosphorylation, we have prepared ERK2 in all phosphorylated forms and kinetically characterized them using two protein substrates, the myelin basic protein and Elk-1. Our results revealed that the catalytic efficiencies of ERK2/pY and ERK2/pT are ~2–3 orders of magnitude higher than that of the unphosphorylated ERK2 and are only 1–2 orders of magnitude lower than that of the fully active ERK2/pTpy. This raises the possibility that the monophosphorylated ERK2s may have distinct biological roles in vivo.

**EXPERIMENTAL PROCEDURES**

**ERK2 Substrates**—Two protein substrates, the myelin basic protein (MBP, obtained from Sigma, M1891) and the glutathione S-transferase (GST) fusion of Elk-1 C-terminal fragment (residues 307–428) were used to study the kinase activity of ERK2. The bacterial expression vector for GST-Elk-1 (307–428) (pEX-2T-Elk-1 (307–428)) was a generous gift from Dr. Kun-Liang Guan. GST-Elk-1 (307–428) was expressed in Escherichia coli BL21(DE3) and purified according to standard procedures using the affinity matrix glutathione-Sepharose 4B (Amersham Biosciences). The protein was further purified by fast protein liquid chromatography Mono Q column with a 100-ml gradient of 0–300 mM NaCl in 20 mM MOPS, pH 7.4, 1 mM EDTA. Fractions containing GST-Elk-1 (307–428) were collected and concentrated with a Centriprep-10 filtration unit (Amicon). Protein concentration was determined using the Bradford dye binding assay (Bio-Rad) diluted 1:100,000 and stored at -80 °C.

**Protein Phosphatases**—The tyrosine-specific HePTP, the Ser/Thr protein phosphatase PP2Ca, and the dual specificity phosphatase MKP3 were used to prepare the monophosphorylated ERK2/pT and ERK2/pY and to determine the stoichiometry of ERK2 phosphorylation. The expression vector for GST-HePTP (pEX-3X-HePTP5690) was a generous gift from Dr. Brent Zanke. GST-HePTP was expressed in E. coli BL21/DE3 and purified according to standard procedures using the affinity matrix Glutathione-Sepharose 4B. The protein purity was judged to be greater than 90% by SDS-PAGE. The purified protein were made to 20% glycerol and stored at −80 °C.

The kinase activity of various forms of ERK2 was used in this study. The chosen fractions were concentrated with a Centriprep-10 filtration unit (Amicon). Analysis by SDS-PAGE showed that the protein purity was greater than 95%. The protein concentration was determined from absorbance measurement at 280 nm using an absorbance coefficient of 1.1 for 1 mg/ml ERK2. The purified protein were made to 20% glycerol and stored at −80 °C.

**Biophosphorylated ERK2/pTpy**—The plasmid pET-His-ERK2-MEK1(R4F) (a generous gift of Dr. Melanie Cobb) was used to co-express a constitutively active MEK1 and an N-terminal His6-tagged ERK2 in E. coli BL21(DE3). The expression and purification of ERK2/pTpy were carried out following the procedure described by Wilsbacher and Sturgill. The fast protein liquid chromatography step about 3 mg of ERK2/pTpy was obtained from 6 liters of culture.

**Monophosphorylated ERK2/pT**—ERK2/pT was prepared by treating ERK2/pTpy with GST-HePTP. In a 100-μl reaction, 7.8 μM ERK2/pTpy and 0.36 μM GST-HePTP were incubated in 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA (MOPS buffer) at 30 °C. Dephosphorylation of Tyr(p) in ERK2/pTpy by GST-HePTP was monitored by measuring the ERK2 kinase activity. At different incubation time, 1 μl of the reaction mixture was withdrawn and diluted into 200 μl of ice-cold MOPS buffer. Then 5 μl of the diluted solution was added to a 50-μl kinase reaction mixture containing 5 μM MBP, 1 mM [γ-32P]ATP (200 cpm/μmol), and 10 mM MgCl2 to initiate the kinase reaction. After incubating at 30 °C for 10 min, the reaction was terminated by 1.5% phosphoric acid. The ERK2 was assayed using the procedure described under “Kinase Assay” in this section. When no further decrease in kinase activity was observed after GST-HePTP treatment, 200 μl of 50% Ni2+-nitrilotriacetic acid metal affinity slurry was added to the reaction mixture. After incubation at 4 °C for 1 h, the resin was washed with 1 ml of 20 mM Tris, pH 7.9, 500 mM NaCl, and 5 mM imidazole 4 times to remove the GST-HePTP. ERK2/pT was eluted from the resin by 500 μl of 20 mM Tris, pH 7.9, 500 mM NaCl, and 200 mM imidazole. The elute was concentrated, and the buffer was changed to 20 mM MOPS, pH 7.4, 100 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol with a Centriprep-10 filtration unit (Amicon). The purified protein were made to 20% glycerol and stored at −80 °C.

**Monophosphorylated ERK2/pT—ERK2/pT was prepared by treating ERK2/pTpy with PP2Ca. In a 100-μl reaction, 6 μM ERK2/pTpy and 0.2 μM PP2Ca was incubated in the MOPS buffer containing 5 mM MnCl2 at 30 °C. Dephosphorylation of Thr(p) in ERK2/pTpy by PP2Ca was monitored by following the ERK2 kinase activity. At different incubation times, 1 μl of the solution was withdrawn and diluted into 200 μl of ice-cold MOPS buffer. The residual kinase activity was measured as described above. When no further decrease in kinase activity was observed, the protein was collected by centrifugation and analyzed by SDS-PAGE. The protein sample was made to 20% glycerol and stored at −80 °C.

**Monophosphorylated ERK2/pT—ERK2/pT was prepared by treating ERK2/pTpy with PP2Ca. In a 100-μl reaction, 6 μM ERK2/pTpy and 0.2 μM PP2Ca was incubated in the MOPS buffer containing 5 mM MnCl2 at 30 °C. Dephosphorylation of Thr(p) in ERK2/pTpy by PP2Ca was monitored by following the ERK2 kinase activity. At different incubation times, 1 μl of the solution was withdrawn and diluted into 200 μl of ice-cold MOPS buffer. The residual kinase activity was measured as described above. When no further decrease in kinase activity was observed, the protein was collected by centrifugation and analyzed by SDS-PAGE. The protein sample was made to 20% glycerol and stored at −80 °C.

**Kinase Assay**—The kinase activity of various forms of ERK2 was assayed by a radioisotope assay in which the rate of incorporation of 32P from [γ-32P]ATP into a substrate was directly measured. Reactions were carried out in 50 mM MOPS buffer containing 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, 1 μM [γ-32P]ATP (PerkinElmer Life Sciences; BLU502A) (200 cpm/μmol), and varied concentrations of the protein substrate. Reactions were initiated by the addition of ERK2 (1 μM), ERK2/pTpy (5–70 nM), ERK2/pT(2–9 nM), or ERK2/pTpy (0.7 nM) and allowed to proceed at 30 °C for 10 min for the phosphorylated ERK2s and 60 min for the unphosphorylated ERK2. The reactions were terminated by the addition of 10 μl of 0.9% (final 1.5%) phosphoric acid. The 32P-labeled product was separated from [γ-32P]ATP using P81 phosphocellulose paper (Whatman, 2.1 cm), which binds to protein or peptide product but not ATP and its metabolites. Details of procedures are as follows. 30 μl of the quenched reaction mixture were spotted onto the 2.1-cm-sized P81 paper strips. After washing the strips with 0.5% phosphoric acid 4 times (2 min each, 10–15 ml of 0.5% phosphoric acid per paper strip) with gentle agitation followed by 1 wash with water and 1 wash with acetone, the P81 papers were dried with a hair dryer and inserted into a 4-mL scintillation tube. Three ml of scintillation liquid was added, and the incorporation of 32P into the product was counted by liquid scintillation spectrometry. Controls were carried out in which ERK2 and the substrate were replaced by buffer. Each sample was measured in triplicate.

**ATPase Assays**—A radioisotope assay (22) was used to determine the ATase activity of unphosphorylated ERK2. Reactions were performed in 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2 in a total volume of 20 μl containing varied concentrations of [γ-32P]ATP (600 cpm/μmol). Reactions were initiated by the addition of 1 μM unphosphorylated ERK2, allowed to proceed at 30 °C for 1 h, then termi-
nated in 1 ml of 0.1 M HCl. To determine the amount of phosphate produced, the quenched reactions were incubated with 200 μl of charcoal solution (10% charcoal (Sigma C-6289), 10% acetic acid, 2.5 mM KH₂PO₄) for 1 h on ice and then centrifuged at maximum speed in a microcentrifuge for 30 min. Radioactivity in the supernatant (500 μl) was determined by a scintillation counter.

The ATPase activities of unphosphorylated ERK2, ERK2/pY, and ERK2/pT/P were also determined using an enzyme-coupled spectrometric assay (24) and a modified inorganic phosphate assay (25).

For the enzyme-coupled spectrometric assay, the coupling reagents (all from Sigma) and their concentrations were 70.4 units/ml lactate dehydrogenase, 25.2 units/ml pyruvate kinase, 1 mM phosphoenolpyruvate, and 10 mM MgCl₂ in a total volume of 250 μl at 30 °C containing varied concentrations of ATP. Reactions were initiated by the addition of ERK2 (3 μM), ERK2/pT (0.5 μM), ERK2/pY (0.7 μM), or ERK2/pT/P (0.07 μM). The rate of phosphate release form ATP was determined by the decrease in absorbance at 340 nm in a 96-well plate using a molar extinction coefficient of 6220 m⁻¹ cm⁻¹.

For the inorganic phosphate assay, reactions were performed in 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl₂ in a total volume of 250 μl at 30 °C containing varied concentrations of ATP. Reactions were initiated by the addition of ERK2 (1.7 μM), ERK2/pT (0.4 μM), ERK2/pY (0.6 μM), or ERK2/pT/P (0.1 μM). The reaction was quenched by the addition of 25 μl of 10% trichloroacetic acid. After the addition of 65 μl of solution (A + B) (A: 2% ammonium molybdate; B: 14% acetic acid; 2% trichloroacetic acid; 125 μl of solution C (2% trisodium citrate-2H₂O plus 2% sodium arsenite in 2% (v/v) acetic acid), the phosphate released form ATP hydrolysis was determined by the absorbance at 700 nm in a 96-well plate using a molar extinction coefficient of 9360 M⁻¹ cm⁻¹.

Phosphorylation States of ERK2 Preparations—Three different methods were used to assess the stoichiometry of ERK2 phosphorylation. (1) The phosphorylation level in ERK2 was determined by the amount inorganic phosphate released from ERK2 upon treatment by various phosphatases. The amount of phosphate produced was followed continuously by a coupled enzyme procedure involving purine nucleoside phosphorylase and its chromophoric substrate 2-amino-6-mercaptopurine ribonucleoside (7-methyl-6-thioguanosine (MTGuo)) (20, 26, 27). Quantitation of phosphate was determined using the extinction coefficient of 11,200 M⁻¹ cm⁻¹ at 360 nm and pH 7.0. The assay was carried out at 25 °C in a 1.6-ml reaction mixture containing 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 0.1 mg/ml purine nucleoside phosphorylase (Sigma), and 50 μM MTGuo. ERK2/pT/P was first treated with HePTP (0.05 μM) and then with MKP3 (0.1 μM) to dephosphorylate Tyr(P) and then Thr(P) on ERK2. The spectrophotometric measurements were conducted using a PerkinElmer Lambda 14 spectrocolorimeter. MTGuo was prepared according to the procedures described by Killilea et al. (27). The extinction coefficient of MTGuo was determined at 331 nm using a molar extinction coefficient of 32,000 M⁻¹ cm⁻¹.

(2) High performance liquid chromatography coupled with mass spectrometry was used to measure the mass of the phosphorylated ERK2s. An HP 1100 high performance liquid chromatography system equipped with a degasser and a binary pump was employed to generate acetonitrile gradient. Solvent A was 5% acetonitrile in deionized water containing 0.1% formic acid, and solvent B was 95% acetonitrile containing 0.1% formic acid. Twenty μM ERK2 samples were diluted 1:10 with deionized H₂O, and 20 μl of the diluted samples were loaded onto a Vydac (Separation Group, Hesperia, CA) 1.0 × 150-mm C4 column. The samples were desalted at 50% solvent B for 20 min and then eluted with a 1-min gradient from 5 to 100% solvent B followed by a 40-min gradient from 100% solvent B. The column effluent (50 μl/min) was monitored continuously using a photodiode array detector and an ion trap mass spectrometer. The mass spectrometer detected the intensity of the ions in the m/z range of 700–1300. (3) The phosphorylated ERK2s were also analyzed by Western blotting experiments. Approximately 10 ng of various forms of ERK2 were loaded on 10% SDS-polyacrylamide gel. When the electrophoresis was complete, the proteins on the gel were transferred to a nitrocellulose membrane using a Trans-Blot SD semidyamid electronic transfer cell (Bio-Rad) at 150 mA and room temperature for 1 h. The proteins on the membrane were probed with anti-ERK1/2 (New England Biolabs, Inc., #9102, 1:2000 dilution), anti-bisphosphorylated ERK1/2 (New England Biolabs, Inc., #9101S, 1:2000 dilution), and anti-phospho-seryl/myrosine-specific antibodies. The immunocomplexes were detected by chemiluminescence upon incubation with ECL reagents (Amersham Biosciences). The membrane was immediately exposed to Kodak BioMax Light Film.

RESULTS

As discussed above, the phosphorylation status of ERK2 is dependent on the balanced activities between ERK2 kinases and phosphatases. Because of the intrinsic catalytic properties of MEK1 and Sert/Thr-specific and Tyr-specific phosphatases, all forms of ERK2, i.e. unphosphorylated ERK2, monophosphorylated ERK2/pT and ERK2/pY, and bisphosphorylated ERK2/pT/P, exist in vivo. However, only the bisphosphorylated ERK2 has been characterized and shown to possess high kinase activity. Although the kinase activities of monophosphorylated ERK2s have not been accurately measured, they are assumed to be inactive and similar to that of the unphosphorylated ERK2. Do monophosphorylated ERK2s have any biological roles in the cell? To begin to address this question, it is important to biochemically characterize all forms of ERK2. In this study we have prepared sufficient quantities of ERK2, ERK2/pT, ERK2/pY, and ERK2/pT/P. This enabled us to fully characterize the kinetic properties of all forms of ERK2s using two different proteins and ATP as substrates.

Preparation of ERK2, ERK2/pT, ERK2/pY, and ERK2/pT/P—The N-terminally (His₆)₅-tagged ERK2 was expressed in E. coli and purified by Ni²⁺-nitrilotriacetic acid chromatography followed by an anion exchange Mono Q fast protein liquid chromatography column. Similar to observations made by others (21, 22), two protein peaks corresponding to ERK2 were found in the anion exchange chromatogram. Because previous structural and biochemical studies of ERK2 were based on fractions from peak 1 (21, 22, 29), we decided to focus on peak 1 as well. We measured the kinase activity of each fraction from peak 1, and as observed by others (22), found that the latter fractions displayed significantly higher activity than those eluting earlier. The higher specific activity associated with the later fractions may be a result of ERK2 autophosphorylation during its induction and expression in E. coli (30). Consequently, only early fractions free of the higher kinase activity were pooled and used in this study, as was an early study (22). ERK2/pT/P was prepared by co-expression of the N-terminal His₆-tagged ERK2 and a constitutively active MEK1 encoded on a single plasmid in E. coli and followed by purification using Ni²⁺-nitrilotriacetic acid affinity and Mono Q anion exchange chromatography (7, 23). To prepare monophosphorylated ERK2/pT, bisphosphorylated ERK2/pT/P was treated with tyrosine-specific HePTP (see “Experimental Procedures”). The extent of Tyr(P) dephosphorylation in ERK2/pT/P by HePTP was followed by measuring the residual kinase activity in ERK2. As expected, HePTP treatment of ERK2/pT/P led to a dramatic decrease in ERK2 kinase activity. The reaction was followed until no further decrease in ERK2 kinase activity was observed to ensure complete tyrosine dephosphorylation (Fig. 1A). We showed previously by phosphoamino acid analysis that exhaustive dephosphorylation of ERK2/pT/P by HePTP or PP2A produced monophosphorylated ERK2/pT or ERK2/pY, respectively, (31). Similarly, monophosphorylated ERK2/pY were prepared by treating ERK2/pT/P with the serine/threonine-specific PP2Cα (Fig. 1B), as described under “Experimental Procedures.”

Physical and Biochemical Characterization of ERK2, ERK2/pT, ERK2/pY, and ERK2/pT/P—To characterize the molecular properties of various forms of ERK2, we first examined the purity of the preparations by SDS-polyacrylamide gel electrophoresis (Fig. 2). In accord with previous observations, monophosphorylated ERK2s co-migrated on SDS-PAGE and could be resolved from the unphosphorylated ERK2 due to gel mobility retardation. As shown in Fig. 2, ERK2, ERK2/pT, and
phosphorylated ERK2, and anti-phosphotyrosine antibodies (Fig. 3). When probed with an anti-ERK2 antibody, all forms of ERK2 showed immunoreactivity. As expected, all phosphorylated ERK2s, i.e. ERK2/pTpY, ERK2/pT, and ERK2/pY, showed similar gel mobility, which was retarded when compared with that of ERK2, consistent with the Coomassie staining in Fig. 2. Note that a faint ERK2 band is also visible in the ERK2/pY sample (Fig. 3, upper panel), supporting the assignment of this band to unphosphorylated ERK2. In every case, treatment of the phosphorylated ERK2s by an appropriate phosphatase (i.e. ERK2/pTpY by MKP3 (an ERK2-specific dual specificity phosphatase (31)), ERK2/pT by PP2Ca, and ERK2/pY by HePTP) produced unphosphorylated ERK2. When probed with an anti-bisphosphorylated ERK2 antibody, only ERK2/pTpY and ERK2/pT displayed immunoreactivity (Fig. 3, middle panel). Only after prolonged exposure can we observe a weak signal for the ERK2/pY sample (data not shown). No measurable immunoreactivity was apparent with the phosphatase-treated ERK2s, indicating that the dephosphorylation was complete. Finally, when probed with an anti-Tyr(P) antibody, only ERK2/pTpY and ERK2/pY show immunoreactivity, as expected (Fig. 3, lower panel). Because similar reactivity toward the anti-Tyr(P) antibody was observed for ERK2/pY and ERK2/pTpY samples, the Tyr(P) levels in both samples were probably similar. Thus, the very weak reactivity of ERK2/pY toward the anti-bisphosphorylated ERK2 antibody was probably due to the intrinsic property of ERK2/pY and not due to a lower Tyr(P) level in the sample. The absence of immunoreactivity in the ERK2/pT sample toward anti-Tyr(P) antibodies indicated that there were no ERK2/pTpY or ERK2/pY present in the sample.

We next directly and quantitatively measured the phosphorylation states of the ERK2 preparations. Two different methods were used to determine the stoichiometry of ERK2 phosphorylation. In the first, the phosphorylation level in ERK2 was determined by the amount of inorganic phosphate released from ERK2 upon treatment by various phosphatases. The amount of phosphate produced was monitored continuously by a coupled enzyme procedure involving purine nucleoside phosphorylase and its chromophoric substrate, MTGuo (26, 27). Quantitation of the phosphate released from phosphorylated ERK2 was determined using the extinction coefficient of 11,200 M⁻¹ cm⁻¹ at 680 nm and pH 7.0. As shown in Fig. 4A, the ratio of Tyr(P) (determined from HePTP treatment) to Thr(P) (determined from MKP3 treatment) in the ERK2/pTPY preparation was 0.9–1.0. Because there were no unphosphorylated ERK2 and other impurity proteins in the ERK2/pTPY sample as judged from SDS-PAGE (Fig. 2), Western blot (Fig. 3), and mass spectrometric analyses (Fig. 5, see below), this result indicated that the ERK2/pTPY sample contains 10% ERK2/pT. Incubation of the monophosphorylated ERK2/pT with HePTP did not produce any inorganic phosphate, consist-
ent with that there were no ERK2/pY in the sample, whereas stoichiometric amounts of phosphate was released from ERK2/pT upon the addition of MKP3 (Fig. 4B). The level of Tyr(P) in ERK2/pY was determined by the amount of phosphate released upon HePTP treatment, which corresponded to 90% of the protein concentration (Fig. 4C). Further treatment with MKP3 did not yield additional phosphate, indicating that there were no ERK2/pT in the sample. The rest of the 10% must be ERK2, which was derived from ERK2/pT in ERK2/pTpY upon PP2Cα treatment.

In the second method, the stoichiometry of ERK2 phosphorylation was examined by electrospray mass spectrometry. To calculate the theoretical mass of ERK2 encoded by the plasmid pET-His6-ERK2-MEK1(R4F), we sequenced both the 5′- and 3′-ends of the ERK2 DNA sequence. The 3′-end sequence was found to be identical to the published data (32). The 5′-end DNA sequence and the corresponding amino acid sequence containing the His6 tag were determined as the following.

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5′-atg cac cac cat cac cat cac gac atg ggc ggc ggc 
M A H H H H H H H A A A 
qgc ggc gcc ccg aqg atg gtc ggc ggg cag gta 3′
A A G P E M V R G Q V24
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Thus, the calculated mass of ERK2 based on the amino acid sequence using the average residue mass is 42297.3. The deconvoluted mass spectra obtained for the ERK2/pTpY, ERK2/pT, ERK2/pY, and ERK2 samples are shown in Fig. 5. After we thoroughly characterized the physical and biochemical properties of various forms of ERK2, we set out to measure their kinase as well as ATPase activities. We selected two proteins, the MBP and the transcription factor Elk-1, as ERK2 substrates. MBP is a widely used protein substrate for several protein kinases, including ERK2 (22). The transcription factor Elk-1 is a physiological substrate of ERK2. When phosphorylated by ERK2, Elk-1 forms a complex with the serum response factor and binds the serum response promoter element to enhance transcription from the c-fos promoter. In this study we used the C-terminal fragment of Elk-1 (residues 307–428) fused to the C terminus of GST. The C-terminal fragment of Elk-1 (residues 307–428) contains all the ERK2 phosphorylation sites (33), and GST-Elk-1-(307–428) has been shown to be an excellent substrate for ERK2 (34).

The kinase activity of ERK2 in various phosphorylation states was determined by a radioisotope assay in which the rate of incorporation of 32P from [γ-32P]ATP into a substrate was directly measured (see “Experimental Procedures”). All steady-state kinetic measurements were performed at pH 7.0 and 30 °C in 1 mM ATP, which is within the range of physiological ATP concentrations. As shown in Fig. 6, the phosphorylation of MBP and GST-Elk-1-(307–428) catalyzed by ERK2/pTpY obeyed classical Michaelis-Menten kinetics. There was no measurable 32P incorporation into GST when GST alone was incubated with ERK2/pTpY and [γ-32P]ATP (data not shown). Similarly, MBP and GST-Elk-1-(307–428) phosphorylation by ERK2, ERK2/pT, and ERK2/pY also followed Michaelis-Menten kinetics. We were able to determine the kinetic parameters, $k_{cat}$ and $K_m$, for all four forms of ERK2 with both MBP (Table II) and GST-Elk-1-(307–428) (Table III) as a substrate. The $k_{cat}$ and $K_m$ for the ERK2/pTpY-catalyzed MBP phosphorylation were 6.51 ± 0.43 s⁻¹ and 10.0 ± 1.3 μM, respectively. Under the same conditions, the $k_{cat}$ and $K_m$ for ERK2 were 0.000728 ± 0.000010 s⁻¹ and 22.9 ± 1.0 μM, respectively. These results were similar to those obtained from earlier studies (measured at pH 7.4 and 23 °C) in which the $k_{cat}$ and $K_m$ for the ERK2/
Adapted from Figure 4, phosphorylation stoichiometry of various phosphorylated forms of ERK2 determined by an enzyme-coupled assay that measures the amount of phosphate released from ERK2 upon treatment with an appropriate phosphatase. The phosphate release from ERK2 was monitored by the increase in absorbance at 360 nm in the presence of 50 μM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA at 360 nm in the presence of 50 μM (35), whereas the \( k_{\text{cat}} \) and \( K_m \) for the unphosphorylated ERK2 were determined to be 0.000151 ± 0.000009 s\(^{-1}\) and 22.4 ± 6.8 μM (22). With GST-Elk-1-(307–428) as a substrate, we obtained a \( k_{\text{cat}} \) of 10.22 ± 0.54 s\(^{-1}\) and \( K_m \) of 1.95 ± 0.20 μM for ERK2/pTpY. These compared with a \( k_{\text{cat}} \) of 1.67 ± 0.33 s\(^{-1}\) and \( K_m \) of 1.5 ± 0.5 μM for ERK2/pY (purchased from New England Biolabs) with the same GST-Elk-1-(307–428) as a substrate at a 10-fold lower (100 μM) substraturation ATP concentration (34).

As shown in Tables II and III, both ERK2/pT and ERK2/pY exhibited dramatically higher kinase activity than that of the unphosphorylated ERK2. For ERK2/pT, the overall substrate turnover number \( k_{\text{cat}} \) for MBP phosphorylation was 620-fold higher than that of ERK2 and only 14-fold lower than that of the fully active ERK2/pTpY. The overall catalytic efficiency, also known as substrate specificity constant \( k_{\text{cat}}/K_m \), was 946-fold higher than that of ERK2 and only 22-fold lower than that of the fully active ERK2/pTpY. Further, phosphorylation at Tyr-185 alone (ERK2/pY) resulted in a 227- and 334-fold increase in \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \), respectively, over those of ERK2. Interestingly, a recent study showed that a constitutively active mutant ERK2, ERK2-L73P/S151D, which was 90% monophosphorylated at Tyr-185 and 10% unphosphorylated, displayed 50–100-fold increase in kinase activity with MBP as a substrate (36).

With GST-Elk-1-(307–428) as a substrate (Table III), the \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) for ERK2/pT were 126- and 368-fold higher than those of ERK2 and only 11- and 20-fold lower than those of ERK2/pTpY. For ERK2/pY, the \( k_{\text{cat}} \) and \( k_{\text{cat}}/K_m \) were 40- and 91-fold higher than those of ERK2 and 34- and 82-fold lower than those of ERK2/pTpY. Collectively, the results from both substrates indicate that the catalytic efficiency of ERK2/pY, ERK2/pT, and ERK2/pTpY are ~2, 3, and 4 orders of magnitude higher than that of ERK2.

We next determined the ATPase activity for all forms of ERK2. For the phosphorylated ERK2s, two different methods were used to measure the ATPase activity. The first was an enzyme-coupled spectrometric assay (24) that measures the ADP produced from ATP hydrolysis. The second was a modified colorimetric inorganic phosphate assay (25) that measures the inorganic phosphate produced from ATP hydrolysis. The ATPase activity of unphosphorylated ERK2 was also determined using a radioisotope assay (22) that measures directly \(^{32}\text{P}\)-labeled inorganic phosphate hydrolyzed from \(^{32}\text{P}\)-ATP in addition to the enzyme-coupled spectrometric assay and the colorimetric inorganic phosphate assay. Similar results were obtained for each form of ERK2 regardless of the method used for the kinetic measurement (Table IV). The \( k_{\text{cat}} \) and \( K_m \) for the hydrolysis of ATP by ERK2/pTpY are comparable with those determined by (22) at pH 7.4 and 23 °C. However, the \( k_{\text{cat}} \) and \( K_m \) for the ATPase activity of the unphosphorylated ERK2 determined at pH 7.4 and 23 °C were 0.00111 ± 0.00009 s\(^{-1}\) and 306 ± 47 μM, respectively, which are 27-fold higher and 5-fold lower than those determined by Prowse and Lew (\( k_{\text{cat}} = 0.0025 ± 0.0003 \text{ min}^{-1} \) and \( K_m = 1.6 ± 0.5 \text{ mM} \)) (22). The source of this discrepancy is currently unknown, but we noted that the ERK2-catalyzed ATP hydrolysis exhibited significant substrate (ATP) inhibition with an apparent \( K_i \) of 4 mM. As summarized in Table IV, phosphorylation of Tyr-185 alone (ERK2/pY) resulted only in a 2-fold increase in the ATPase activity. In contrast, phosphorylation of Thr-183 alone

\[
v = \frac{V_{\text{max}}}{1 + \frac{[S]}{K_m} + \frac{[S]}{K_i}},
\]  
(Eq. 1)
(ERK2/pT) led to a 16-fold increase in the ATPase activity. Phosphorylation of both Thr-183 and Tyr-185 (ERK2/pTpY) increased the ATPase activity by 62-fold. It should be kept in mind that because the kinase activity of ERK2/pT is 11–14-fold lower than that of ERK2/pTpY, the small amount (≤10%) of ERK2/pT in the ERK2/pTpY sample do not affect the kinetic parameters determined for ERK2/pTpY. Indeed, we have recently obtained a new batch of bisphosphorylated ERK2 that is free of ERK2/pT. The newly prepared ERK2/pTpY displayed similar kinetic properties to those reported in this study.3 Similarly, because the activity of ERK2 is much lower than that of ERK2/pY, the small amount of ERK2 in the ERK2/pY sample is insignificant to alter the kinetic properties of ERK2/pY.

Is the Observed High Kinase Activity in the Monophosphorylated ERK2s Due to a Small Amount of Contaminating Bisphosphorylated ERK2? In addition to the data presented (phosphoamino acid analysis (31), specific phosphatase treatments (Figs. 1 and 4), Western blot analysis (Fig. 3), and mass spectrometry (Fig. 5)), three additional lines of evidence suggest that the high kinase activity observed for the monophosphorylated ERK2s is intrinsic to ERK2/pY and ERK2/pT, not from contaminating ERK2/pTpY. First, when the purified ERK2/pT and ERK2/pY, which were derived from treatment of ERK2/pTpY by HePTP and PP2C, respectively, were treated with fresh, additional HePTP or PP2Ca, no further decrease in ERK2 kinase activity was observed. Second, we have established, based on Western blot analysis with anti-Tyr(P) antibodies, that if the ERK2/pT sample contains ERK2/pTpY, its content must be less than 0.1% of the total protein content. This amount cannot account for the $k_{cat}$ values determined for ERK2/pT, which amount to 6.9–9.2% that of ERK2/pTpY. Finally, if the observed activity in the monophosphorylated samples were due to the more active ERK2/pTpY, then the $K_m$ should reflect that of ERK2/pTpY. As can be seen in Tables II-IV, the $K_m$ values of ERK2/pT and ERK2/pY are substantially different from those of ERK2/pTpY.

To exclude the possibility that the observed higher kinase activity of the monophosphorylated ERK2s was originated from bisphosphorylated ERK2 resulting from autophosphorylation of the monophosphorylated forms of ERK2 during the kinase reaction, we determined the level of autophosphorylation in ERK2, ERK2/pT, ERK2/pY, and ERK2/pTpY in the presence of Mg$^{2+}$-[γ-32P]ATP (Fig. 7). It is known that ERK2 can autophosphorylate weakly at Thr-183, Tyr-185 (30, 37, 38), and Ser-39 (38), and the mechanism of ERK2 autophosphorylation is intramolecular (36). The stoichiometry of autophosphorylation is generally less than 1% but could approach 15–20% after extended incubation with Mg$^{2+}$-ATP. Autophosphorylation of ERK2 was slow, and the increase in ERK2 kinase activity was small (less than 1%) compared with that occurring in the presence of ERK2 kinase activator (38). As shown in Fig. 7, within the incubation time for kinase activity measurements (10 min for the phosphorylated ERK2s and 60 min for unphosphorylated ERK2), there was no significant autophosphorylation for ERK2/pY, ERK2/pTpY, and ERK2. Interestingly, ERK2/pT displayed the highest autophosphorylation activity. We determined that the stoichiometry of ERK2/pT autophosphorylation reached 14% after a 10-min incubation with Mg$^{2+}$-[γ-32P]ATP. However, autophosphorylation of ERK2/pT did not alter its kinase activity (Table V). Furthermore, Western blot analysis

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3 S. Reddy, unpublished results.

### Table I

The mass of various forms of ERK2 determined by mass spectrometry

| Mass          | Expected mass difference (Da) | Observed mass difference (Da) |
|---------------|-------------------------------|------------------------------|
| ERK2          | 42,164                        | 0                            |
| ERK2/pT       | 42,243                        | 81                           |
| ERK2/pY       | 42,243                        | 81                           |
| ERK2/pTpY     | 42,322                        | 162                          |

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**Fig. 5.** Mass spectra of ERK2, ERK2/pT, ERK2/pY, and ERK2/pTpY. The biggest error limit for the mass measurements was within ±5 daltons. ERK2 was derived from ERK2/pTpY treated with MKP3. For preparation of ERK2/pT and ERK2/pY, see “Experimental Procedures.”
Regulation of MAP Kinase Activity by Differential Phosphorylation

Monophosphorylation in the Activation Loop Produces Intermediate Activity States in ERK2—The activities of many protein kinases, which catalyze protein phosphorylation reactions, are themselves regulated by phosphorylation (4). Most protein kinases are activated through phosphorylation of amino acid residue(s) within the activation loop. Full activation of MAP kinases requires the phosphorylation of both Thr and Tyr in the TXY motif in the activation loop. It has been shown that MEK1 carries out its dual phosphorylation of ERK2 by a distributive mechanism (13, 14). Interestingly, the dephosphorylation of ERK2/pTpY by MKP3 is also distributive (31). In addition, because bisphosphorylated MAP kinases contain both Thr(P) and Tyr(P), they could serve as substrates for all classes of protein phosphatases. Thus, dephosphorylation of the bisphosphorylated MAP kinase by serine/threonine protein phosphatases should produce MAP kinase phosphorylated on Tyr, whereas dephosphorylation of the bisphosphorylated MAP kinase by protein-tyrosine phosphatases should yield MAP kinase phosphorylated only on Thr. Consequently, it is expected that monophosphorylated MAP kinases would be generated through the combined action of MEKs and various MAP kinase phosphatases. Indeed, recent evidence indicates that both forms of the monophosphorylated ERK2 exist in the cell in addition to the bisphosphorylated and unphosphorylated ERK2 (18, 19). However, it is widely accepted in the literature that only the bisphosphorylated MAP kinase is active, whereas the monophosphorylated MAP kinases are inactive. Thus, the biological roles (if any) for the monophosphorylated MAP kinases are currently unknown.

Do monophosphorylated MAP kinases have biological functions in vivo? To begin to address this question, we need to biochemically characterize all forms of MAP kinase. Using previously published procedures, we obtained recombinant ERK2 and ERK2/pTpY. We then prepared ERK2/pT and ERK2/pY by treating ERK2/pTpY with tyrosine-specific HEPTP and serine/threonine-specific PP2Ca, respectively. We determined the kinetic parameters for the phosphorylation of both MBP and Elk-1 catalyzed by all forms of ERK2. Our results revealed that the kinase activity and catalytic efficiency of ERK2/pY and ERK2/pT are only 1–2 orders of magnitude lower than those of the bisphosphorylated ERK2. More importantly, the kinase activity and catalytic efficiency of ERK2/pY and ERK2/pT are 2–3 orders of magnitude higher than those of the unphosphorylated ERK2 (Fig. 8). Thus, our results show that monophosphorylation in the activation loop produces intermediate activity states in ERK2. Phosphorylation of Tyr-185 increases the overall catalytic efficiency by 2 orders of magnitude, and phosphorylation of Thr-183 increases the overall catalytic efficiency by nearly 3 orders of magnitude. Dual phosphorylation of Thr-183 and Tyr-185 increases the overall catalytic efficiency by 4 orders of magnitude. It is important to point out that the kinetic properties for ERK2/pY and ERK2/pT reported here are different from those measured for ERK2/T183A/pY and ERK2/Y185F/pT (38, 39), indicating that ERK2/T183A/pY and ERK2/Y185F/pT are not accurate models for the native monophosphorylated ERK2s.

Structural and Functional Correlation—The three-dimensional structures for the low activity, unphosphorylated ERK2, and the fully active bisphosphorylated ERK2/pTpY were solved, and a comparison of the two structures suggested a structural basis of ERK2 activation induced by the dual phosphorylation in the activation loop (7, 29). Like many other protein kinases, the activation loop in ERK2 plays a critical role in modulating its activity. In inactive ERK2, the two lobes in the kinase domain exist in an open conformation, and the conformation of the activation loop is incompatible with substrate binding and/or catalysis. ERK2 activation is triggered by a conformational change that is initiated by dual phosphorylation of Thr-183 and Tyr-185 within the activation loop. Phosphorylation of Tyr-183 promotes rotation of the two lobes toward each other, and the phosphate on Tyr-183 makes ionic interactions with the substrate that favors catalysis (15). Phosphorylation of Thr-183 further supports the interaction between the two lobes, and the resulting conformation promotes catalysis. These findings provide a structural basis for the activation of ERK2 through dual phosphorylation.
contacts with three Arg residues, Arg-68 in helix C, Arg-146 in the catalytic loop, and Arg-170 in the activation loop. One of the major consequences of these interactions is the optimization of the alignment of the invariant residues, Lys-52, which coordinates to the α- and β-phosphates of ATP, and Glu-69, which stabilizes Lys-52 in ERK2/pTpY. Mutagenesis studies of Lys-52 indicate that its primary function is to facilitate phosphoryl group transfer (40). In addition, domain rotation brings Lys-52 and the phosphate binding loop closer to Asp-147, which is the catalytic base that accepts a proton from the hydroxyl group of substrate Ser. and Asp-165, which plays a role in Mg2+ ion coordination. Tyr-185 is buried and inaccessible to solvent in unphosphorylated ERK2. Upon Tyr-185 phosphorylation, the activation loop is refolded, which enables the phosphate on Tyr-185 to interact with Arg-189 and Arg-192, leading to the formation of the P–Tyr-185 to interact with Arg-189 and Arg-192, leading to the formation of the P–tyrosine. ERK2/pT was incubated with [γ-32P]ATP, and at the indicated time, an aliquot of ERK2/pT was withdrawn, and its kinase activity measured using MBP as a substrate.

**TABLE II** Kinetic parameters of ERK2 in different phosphorylation states with MBP as a substrate

|        | Km  | kcat | Relative kcat | hcat/Km | Relative hcat/Km |
|--------|-----|------|--------------|---------|-----------------|
| ERK2/pTpY | 10.4 ± 1.3 | 6.51 ± 0.43 | 8940 | (6.51 ± 0.90) × 10^5 | 20,540 |
| ERK2/pT | 15.2 ± 2.4 | 0.451 ± 0.027 | 620 | (3.00 ± 0.45) × 10^4 | 946 |
| ERK2/pY | 15.5 ± 2.1 | 0.165 ± 0.016 | 227 | (1.06 ± 0.13) × 10^4 | 334 |
| ERK2   | 22.9 ± 1.0 | (7.28 ± 0.10) × 10^3 | 1.0 | 31.7 ± 1.4 | 1.0 |

**FIG. 7.** Autophosphorylation of ERK2 (○), ERK2/pY (▼), ERK2/pT (■), and ERK2/pTpY (▲). The level of autophosphorylation in ERK2, ERK2/pY, ERK2/pT, or ERK2/pTpY was determined under the following conditions. 1.1 µM phosphorylated or unphosphorylated ERK2 was incubated with 1 mM [γ-32P]ATP (200 cpm/pmol) and 10 mM MgCl2 in 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA at 30 °C. At different incubation times, 50 µl of the reaction mixture was withdrawn and mixed with 10 µl of 9% phosphoric acid to terminate the autophosphorylation reaction. The incorporated 32P in ERK2 was measured by filter binding and scintillation counting.

**TABLE III** Kinetic parameters of ERK2 in different phosphorylation states with GST-Elk-1/307–428 as a substrate

|        | Km  | kcat | Relative kcat | hcat/Km | Relative hcat/Km |
|--------|-----|------|--------------|---------|-----------------|
| ERK2/pTpY | 1.95 ± 0.20 | 10.22 ± 0.54 | 1370 | (5.24 ± 0.53) × 10^6 | 7450 |
| ERK2/pT | 3.63 ± 0.35 | 0.939 ± 0.033 | 126 | (2.59 ± 0.25) × 10^5 | 368 |
| ERK2/pY | 4.74 ± 0.79 | 0.302 ± 0.020 | 40.5 | (6.37 ± 1.07) × 10^4 | 90.6 |
| ERK2   | 10.6 ± 1.4 | (7.46 ± 0.50) × 10^3 | 1.0 | 703.3 ± 95.0 | 1.0 |

**TABLE IV** ATPase activity of ERK2 in different phosphorylation states

|        | Km  | kcat | Relative kcat | hcat/Km | Relative hcat/Km |
|--------|-----|------|--------------|---------|-----------------|
| ERK2/pTpY | 88.3 ± 7.7 | 0.135 ± 0.004 | (1.52 ± 0.13) × 10^3 | Inorganic phosphate |
| ERK2/pT | 82.0 ± 7.8 | 0.127 ± 0.003 | (1.55 ± 0.15) × 10^3 | Coupled spectrophotometric |
| ERK2/pY | 127 ± 15.8 | 0.0333 ± 0.0015 | 262 ± 33 | Inorganic phosphate |
| ERK2   | 138 ± 33.2 | 0.0343 ± 0.0025 | 248 ± 59 | Coupled spectrophotometric |
| ERK2/pT | 145 ± 26.5 | 0.00425 ± 0.00028 | 293 ± 3.4 | Inorganic phosphate |
| ERK2/pY | 162 ± 52.3 | 0.00428 ± 0.00048 | 30.1 ± 9.8 | Coupled spectrophotometric |
| ERK2   | 289 ± 45.7 | 0.00286 ± 0.00017 | 9.89 ± 1.57 | Radiosotope |
| ERK2/pT | 326 ± 77.9 | 0.00162 ± 0.00020 | 4.97 ± 1.19 | Inorganic phosphate |
| ERK2/pY | 294 ± 24.2 | 0.00186 ± 0.00006 | 6.32 ± 0.52 | Coupled spectrophotometric |

**TABLE V** Kinetic parameters of ERK2/pT as a function of pre-incubation time with Mg2+-ATP

| Pre-incubation time | Km  | kcat | Relative kcat | hcat/Km | Relative hcat/Km |
|---------------------|-----|------|--------------|---------|-----------------|
| 5 µM                | 15.5 ± 2.0 | 0.394 ± 0.019 | (2.55 ± 0.38) × 10^4 | - |
| 10 µM               | 15.2 ± 3.2 | 0.451 ± 0.027 | (2.97 ± 0.45) × 10^4 | - |
| 20 µM               | 15.5 ± 1.2 | 0.373 ± 0.013 | (2.41 ± 0.21) × 10^4 | - |
| 40 µM               | 14.3 ± 2.3 | 0.446 ± 0.025 | (3.12 ± 0.51) × 10^4 | - |
| 60 µM               | 16.1 ± 2.3 | 0.453 ± 0.024 | (2.81 ± 0.41) × 10^4 | - |
ATPase activity that is 62-fold higher than that of the unphosphorylated ERK2. Interestingly, phosphorylation of Tyr-183 alone results in a 16-fold increase in the ATPase activity. Moreover, the kinase activity of ERK2/pT is 2–3 orders of magnitude higher than that of the unphosphorylated ERK2 and only 11–14-fold lower than that exhibited by ERK2/pTpY. These results are consistent with the structural observations that Tyr-183 phosphorylation orchestrates the correct positioning of the active site for efficient phospho-transfer. The fact that the extent of activation in the ATPase reaction is significantly less than that observed in the kinase reaction suggests that Tyr-183 phosphorylation contributes to the stabilization of both the ATP moiety and the protein phospho-acceptor substrate in the transition state of the kinase reaction.

Unlike Tyr-183, which is equivalent to Thr-160 in cyclin-dependent kinase 2 and Thr-197 in protein kinase A, Tyr-185 is unique to the MAP kinases. We discovered that phosphorylation of Tyr-185 alone does not have significant effect on the ATPase activity, which is in accord with the structural data that Tyr-185 is not involved in alignment of Lys-52 and Glu-69. Strikingly, phosphorylation of Tyr-185 dramatically augments the rate of phospho-transfer from ATP to protein substrates. The $k_{cat}$ values for the ERK2/pY-catalyzed phosphorylation of MBP and GST-Elk-1 (307–428) are 227- and 40.5-fold higher than those of the unphosphorylated ERK2. In fact, ERK2/pY is only 3-fold less active than ERK2/pT. These results support the notion that phosphorylation of Tyr-185 is responsible for P + 1 site recognition in protein substrates. Because catalytic activation of the ERK2/pY kinase activity results primarily from a large increase in the rate of the phosphoryl transfer from ATP to the protein substrates, phosphorylation of Tyr-185 serves primarily to position the phospho-acceptor for phosphoryl transfer.

**Biological Implications**—Activation of ERK2 is involved in cellular proliferation, transformation, and differentiation. It is well known that both the magnitude and duration of ERK2 activation are important in determining the cell fate (1–3). For example, in PC12 cells, transient induction of ERK2 activity promotes cell proliferation, whereas sustained activation of ERK2 drives cells into neuronal differentiation (41). The only known mechanism for ERK2 activation is through dual phosphorylation of the Thr and Tyr residues in the activation loop by MEK1 (5–7). In fact, the dual phosphorylation of ERK2 has been proposed as an on-off switch, increasing the basal activity by more than 1,000-fold. Intriguingly, ERK2/pTpY can be dephosphorylated by multiple phosphatases, including the Ser/Thr phosphatases, the tyrosine-specific protein-tyrosine phosphatases, and the dual specificity phosphatase MKPs. The action of different phosphatases on ERK2/pTpY would inevitably generate monophosphorylated ERK2s in addition to the unphosphorylated ERK2. However, in much of the literature investigating ERK2 signaling, there has been the implicit assumption that the monophosphorylated ERK2s are inactive. Thus, the significance for the need of multiple phosphatases to down-regulate the ERK2 kinase activity is not clear.

Our observation that a single phosphorylation in the activation loop of ERK2 produces an intermediate activity state challenges the current accepted view that only the bisphosphorylated ERK2 has biological functions. It has been proposed that multi-site phosphorylations could correlate with the generation of a variety of protein forms in which one or more properties are altered (42, 43). For example, different phosphorylations could be linked to distinct protein functions or graded effects on a single function. Our data suggest a more graded change in kinase activity induced by phosphorylation of the ERK2 activation loop than from a simple off versus on switch for kinase activation. Recent studies indicate that significant amounts of monophosphorylated ERK2s exist in vivo and the monophosphorylated ERK2s have distinct subcellular localizations (18, 19). More importantly, results from a constitutive active mutant of ERK2, ERK2-L73P/S151D, suggest that only a 50–100-fold increase in ERK2 activity is sufficient to sustain ERK2 function and establish the threshold of ERK2 activity needed for in vivo signaling (36). Our results that monophosphorylated ERK2s possess kinase activities that are 2–3 orders of magnitude higher than that of the unphosphorylated ERK2 raise the possibility that ERK2/pY and ERK2/pT may indeed have distinct in vivo functions. Although less active than the bisphosphorylated species, monophosphorylated ERK2s may differentially phosphorylate pathway components.

One possibility that the biological importance of the monophosphorylated ERK2s has not been appreciated may be linked to the fact that most assays used for assessing ERK2 activation rely heavily on the use of gel mobility shift and anti-phospho-ERK2 antibodies. As shown in this work and by others (14, 18, 19, 36), one cannot differentiate the bisphosphorylated ERK2 from the monophosphorylated proteins using these methods/reagents. Thus, activities of the monophosphorylated ERK2s may have gone undetected because they were attributed to the bisphosphorylated ERK2.

Finally, because the MAP kinase phosphorylation sites and the machinery for MAP kinase phosphorylation are conserved, it seems plausible that the monophosphorylated forms of other MAP kinases would display intermediate levels of activity as

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**FIG. 8.** Schematic presentation of the relative kinase activity (A) and catalytic efficiency (B) between various forms of ERK2. Open bar, with MBP as a substrate; solid bar, with GST-Elk-1 as a substrate.
well. The fact that multiple phosphatases are involved in the regulation of MAP kinase activity suggests that phosphatases may play a crucial role in determining cellular responses to external stimuli. The involvement of multiple phosphatases for MAP kinase inactivation may also have an impact on the time course, the threshold for activation, and the efficiency of regulation of the MAP kinase pathways.

In summary, this study reveals that monophosphorylated ERK2s have intermediate activities in between the basal unphosphorylated and the fully activated bisphosphorylated ERK2s. The results illustrate the flexibility of phosphorylation and its ability to regulate activity states. This may be important for the ability of ERK2 to integrate diverse biological stimuli and permit more intricate regulatory circuits to operate. In the context of our findings, monophosphorylated forms of ERK2 may have as yet unidentified, distinct biological functions.

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