The extracellular signal-regulated protein kinase 2 (ERK2) is the founding member of a family of mitogen-activated protein kinases (MAPKs) that are central components of signal transduction pathways for cell proliferation, stress responses, and differentiation. The MAPKs are unique among the Ser/Thr protein kinases in that they require both Thr and Tyr phosphorylation for full activation. The dual phosphorylation of Thr-183 and Tyr-185 in ERK2 is catalyzed by MAPK/ERK kinase 1 (MEK1). However, the identity and relative activity of protein phosphatases that inactivate ERK2 are less well-established. In this study, we performed a kinetic analysis of ERK2 dephosphorylation by protein phosphatases using a continuous spectrophotometric enzyme-coupled assay that measures the inorganic phosphate produced in the reaction. Eleven different protein phosphatases, many previously suggested to be involved in ERK2 regulation, were compared, including tyrosine-specific phosphatases (PTP1B, CD45, and HePPT), dual specificity MAPK phosphatases (VHR, MKP3, and MKP5), and Ser/Thr protein phosphatases (PP1, PP2A, PP2B, PP2Ca, and λPP). The results provide biochemical evidence that protein phosphatases display exquisite specificity in their substrate recognition and indicate HePPT, MKP3, and PP2A as ERK2 phosphatases. The fact that ERK2 inactivation could be carried out by multiple specific phosphatases shows that signals can be integrated into the pathway at the phosphatase level to determine the cellular response to external stimuli. Important insights into the roles of various protein phosphatases in ERK2 kinase signaling are obtained, and further analysis of the mechanism by which different protein phosphatases recognize and inactivate MAPKs will increase our understanding of how this kinase family is regulated.

Protein phosphorylation is the most important post-translational modification mechanism utilized by eukaryotic cells to regulate protein functions and is involved in switching cellular activities from one state to another and, in this way, controlling gene expression, cellular proliferation, and cell differentiation. The level of protein phosphorylation inside the cell is controlled by the balanced activities of protein kinases and protein phosphatases. Consequently, protein kinases and phosphatases are key components of intracellular signal transduction pathways through which cells respond to extracellular signals, such as hormones and growth factors, as well as environmental and nutritional stresses.

The mitogen-activated protein kinases (MAPKs) are the major convergence points in these signaling pathways. The three best-characterized MAPK cascades include the extracellular signal-regulated protein kinase (ERK) pathway, which responds to stimuli that induce proliferation and differentiation, the c-Jun N-terminal protein kinase (JNK) pathway, and the p38 kinase pathway, both of which are activated in response to environmental stresses. After activation, each MAPK phosphorylates a distinct spectrum of substrates, which includes key regulatory enzymes, cytoskeletal proteins, regulators of apoptosis, nuclear receptors, and many transcription factors. Such a broad array of substrates is consistent with the observation that MAPKs control many critical cell functions.

Because of the critical importance of MAPK in cellular signaling, the activity of the MAPK is tightly regulated. The MAPK is activated by phosphorylation within a structurally conserved activation loop. However, unlike many other protein kinases, whose activation requires phosphorylation of a single residue within the activation loop (2), MAPKs are activated via a MAPK/ERK kinase (MEK)-catalyzed dual phosphorylation of the Thr and Tyr residues in the TXY motif of the activation loop (where X is Glu in ERKs, Pro in JNKs, and Gly in p38 kinases) (3–8). Although the activation of MAPKs has been extensively studied (9, 10), the equally important process of quenching MAPK activity is poorly understood. Evidence suggests that MAPK inactivation may involve all major types of protein phosphatases (11). Protein phosphatases are broadly classified into two major families: the protein Ser/Thr phosphatases and the protein tyrosine phosphatases (PTPs). Protein Ser/Thr phosphatases remove the phosphoryl group from serine/threonine residues, whereas PTPs dephosphorylate tyrosine residues in phosphoprotein substrates. Structural comparisons of the catalytic do-

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The Specificity of Extracellular Signal-regulated Kinase 2 Dephosphorylation by Protein Phosphatases*
mains of PTPs with the catalytic subunits of protein Ser/Thr phosphatases reveal no similarity. In addition, these two classes of protein phosphatases have evolved to employ completely different strategies to accomplish the dephosphorylation reaction. This is in striking contrast to the situation with protein kinases, where tyrosine-specific and serine/threonine-specific kinases share significant sequence similarity in the catalytic domains and utilize similar structural and mechanistic features for catalysis (2, 12, 13). Thus, although the protein Ser/Thr phosphatases are metalloenzymes with bimetallic centers containing iron and effect catalysis by direct attack of an activated water molecule at the phosphorus atom of the substrate (14), the PTPs do not require metals and proceed activated water molecule at the phosphorus atom of the substrate containing iron and effect catalysis by direct attack of an activated water molecule at the phosphorus atom of the substrate (14), the PTPs do not require metals and proceed

Preparation of Bisphosphorylated ERK2—The plasmid pET-His6-ERK2-MEK1(RA) (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 (ERK2 phosphorylated on both Thr-183 and Tyr-185) were carried out following the procedure described by Wibabacher and Cobb (24). After the final fast-protein liquid chromatography step, about 1–3 mg of ERK2/pTpY were obtained from 6 liters of culture. Electrospay ionization mass spectrometry analysis confirmed that the purified ERK2/pTpY was effectively homogenous and was phosphorylated stoichiometrically to a ratio of 2 mol of phosphate per mole of ERK2.

Preparation of Monophosphorylated ERK2/pT and ERK2/pY—The monophosphorylated ERK2/pT and ERK2/pY were prepared by treating ERK2/pTpY with the tyrosine-specific HePTP and the Ser/Thr protein phosphatase PP2Ca, respectively, as described previously (23, 25).

Phosphatase Activity Measurements with pNPP as a Substrate—Initial rates for the hydrolysis of para-nitrophenyl phosphate (pNPP) by PTP1B, HePTP, CD45, VHR, MKP3, and MKP5 were measured at 30 °C in pH 7.0 buffer containing 50 mM 3,3-dimethylglutarate, 1 mM EDTA, and 1 = 0.15 M, as described previously (26). The activities of the Ser/Thr phosphatases were determined at 30 °C in pH 7.0 buffer containing 50 mM 3,3-dimethylglutarate, 0.1 mM EDTA, and 1 = 0.15 M, in the presence of either 10 mM MgCl2 for PP2A or 2 mM MnCl2 for PP2Ca, PP1, λ-PPase, and PP2B. In addition, for PP2B, the assay mixture also contained 0.5 μM calmodulin.

A Continuous Spectrophotometric Assay for Protein Phosphatases Using a Phosphoprotein as a Substrate—In this study, we prepared ERK2 in its various phosphorylation states. We then performed detailed kinetic measurements to quantitatively examine them as substrates for a number of protein phosphatases implicated in the regulation of ERK2 activity. This has allowed us to identify the protein phosphatases that are most likely the physiological regulators of ERK2 activity. Comparison of the kinetic constants for ERK2 dephosphorylation by these different phosphatases reveals that protein phosphatases display strikingly high substrate specificity for ERK2 and provides important insights into the roles of various protein phosphatases in ERK2 kinase signaling.

EXPERIMENTAL PROCEDURES

**Protein Phosphatases**—Recombinant PTP1B (residues 1–321) (17), VHR (18, 19), and MKP3 (20) were expressed in Escherichia coli and purified as described previously. The MKP5 coding DNA fragment was a generous gift from Dr. Eisuke Nishida. Recombinant MKP5 was expressed in E. coli and purified as a glutathione S-transferase (GST) fusion protein. The catalytic domain of CD45 (containing both D1 and D2) was expressed and purified as a recombinant GST fusion protein (21). The recombinant PP1 was purchased from Sigma Chemical Co. (P-7937). The recombinant PP2B (calcineurin) and the bacteriophage λ protein Ser/Thr phosphatase (AP) were generous gifts from Dr. Frank Rusnak. PP2A was purified from human red blood cells by a modification of the method of Usui et al. (22) with a final step of Mono Q chromatography to obtain the purified AC dimer. The N-terminal His6-tagged HePTP was expressed in E. coli BL21(DE3) and purified using standard procedures of nickel chelate affinity chromatography (23). The human N-terminal His6-tagged PP2Ca was expressed in E. coli BL21/DE3 using the expression vector pET28a-His6-PP2Ca (a generous gift from Dr. Mark Solomon) and purified using standard procedures of nickel chelate affinity chromatography. The protein purity was over 90% as judged by SDS-PAGE. The purified proteins were made to 20% glycerol and stored at −80 °C.

**Data Analysis**—For the HePTP-catalyzed ERK2 dephosphorylation, the phospho-ERK2 concentration greatly exceeded that of HePTP. The kinetic parameters were obtained by fitting the v versus [S] curve to the Michaelis-Menten equation. For MKP3 and PP2Ca, the substrate (phospho-ERK2) concentration was comparable to that of the phosphatase. The kinetic parameters were obtained by fitting the v versus [S] curve to the following initial rate equation (29),

$$v = 0.5 \times k_{cat} \times ([K_m + [E]]_0 + [S]) - ([K_m + [E]]_0 + [S])^2 - 4 \times [E]_0 \times [S]$$

(Eq. 1)

For PTP1B, CD45, VHR, MKP5, PP2A, PP1, PP2B, and AP, the substrate concentration was much lower than the $K_m$. Under this condition, the reaction is first order with respect to [S] and at a given phosphatase concentration, the observed apparent first order rate constant is equal to $k_{cat}/[E]_0$. Thus, the $k_{cat}/[E]_0$ values for these phosphatases were obtained by fitting the progress curves to the equation,

$$P = [S]_0 \times (1 - e^{-k_{cat}[E]_0})$$

(Eq. 2)

In these measurements, the ERK2/pTpY concentration was less than 1 μM, and the concentration of the protein phosphatases ranged from 0.03 to 0.1 μM. For Thr/Ser phosphatases, the reaction mixture also contained an appropriate concentration of metal ion as specified above.
RESULTS AND DISCUSSION

For meaningful and insightful mechanistic studies of protein phosphatases, it is essential to use homogeneous phosphoproteins that correspond to the physiological substrates of the phosphatases. However, there are few detailed mechanistic studies of protein phosphatases with physiological substrates. Probably the major reasons for this are that phosphorylated proteins are notoriously difficult to prepare in sufficient quantities in a highly specific and stoichiometric form and that phosphorylated proteins are also often difficult to assay for accurate kinetic measurements. These difficulties have limited our ability to dissect the role of protein phosphatases in cellular signaling. The most commonly used approaches for studying the substrate specificity of protein phosphatases involve overexpression experiments followed by either monitoring a target reporter gene expression or in vitro assay of kinase activity/phosphorylation level in the immunoprecipitate of cellular extracts. These kinds of experiments are either indirect or non-quantitative, and they do not provide definitive information about the identity of protein phosphatases involved in certain cellular functions. Indeed, one would almost always observe substrate dephosphorylation if sufficient amount of a protein phosphatase is provided to the system. Consequently, numerous protein phosphatases have been proposed as regulators of the MAPK ERK2 based on activity measurements at a single time point and protein concentration. These results give the erroneous impression that there is a relative lack of specificity in substrate dephosphorylation by protein phosphatases.

In this study, we prepared milligram quantities of phosphorylated ERK2s, developed a continuous spectrophotometric enzyme-coupled assay that measures the inorganic phosphate produced in the phosphatase reaction, and analyzed the dephosphorylation of ERK2 by 11 protein phosphatases, many of which were previously suggested as regulators of ERK2 signaling. The results lead to the identification of phosphatases that are likely in vivo regulators of ERK2 signaling and demonstrate that protein phosphatases possess exquisite specificity toward their physiological substrates.

Preparation of ERK2/pTpY, ERK2/pT, and ERK2/pY—The bisphosphorylated ERK2, ERK2/p2pY (ERK2 phosphorylated on both Thr-183 and Tyr-185), was prepared by co-expression of the N-terminal His6-tagged ERK2 and a constitutively active MEK1 encoded on a single plasmid in Escherichia coli. The homogeneity and phosphorylation stoichiometry (1 mol/mol for ERK2/pT and ERK2/pY, and 2 mol/mol for ERK2/p2pY) of the ERK2 preparations were verified by SDS-PAGE and liquid chromatography-mass spectrometry as described previously. The preparations of ERK2/pTpY, ERK2/pT, and ERK2/p2pY—The bisphosphorylated ERK2, ERK2/p2pY (ERK2 phosphorylated on both Thr-183 and Tyr-185), was prepared by co-expression of the N-terminal His6-tagged ERK2 and a constitutively active MEK1 encoded on a single plasmid in Escherichia coli and followed by purification using nickel-nitrilotriacetic acid affinity and Mono Q anion exchange chromatography.

A Continuous Spectrophotometric Enzyme-coupled Assay for Monitoring ERK2 Dephosphorylation by Protein Phosphatases—To facilitate the analysis of the phosphatase-catalyzed ERK2 dephosphorylation, we employed a continuous spectrophotometric enzyme-coupled assay that measures the inorganic phosphate produced in a phosphatase reaction. In this coupled enzyme system, the coupling enzyme, purine nucleoside phosphorylase, uses the inorganic phosphate, generated by the action of the phosphatase, to convert 7-methyl-6-thioguanosine to ribose-1-phosphate (Reactions 1 and 2), resulting in an increase in absorbance at 360 nm. This reaction is essentially irreversible.

The effects of different levels of purine nucleoside phosphorylase on the coupled enzyme system were examined to determine the optimal amount of this enzyme required to couple the HePTP-catalyzed ERK2 dephosphorylation. As expected, at low concentrations of the coupling enzyme, an initial lag phase was observed. The lag phase decreased as the concentration of the coupling enzyme was increased (data not shown). When the concentration of the purine nucleoside phosphorylase was raised above 0.02 mg/ml, the initial lag phase disappeared completely and the linear (steady-state) phase remained unchanged with time. On the basis of these observations, the concentration of the coupling enzyme in the phosphatase assay was set to 0.1 mg/ml, which was more than adequate to rapidly utilize the inorganic phosphate released from the phosphatase reactions. Similarly, the concentration of 7-methyl-6-thioguanosine used in the coupling assay was also optimized. All kinetic parameters for the dephosphorylation of the phosphorylated ERK2 proteins by the protein phosphatases were determined using this continuous spectrophotometric assay.

All experiments were carried out at 25 °C in a 1.6-ml reaction mixture containing 50 mM MOPS at pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 0.1 mg/ml purine nucleoside phosphorylase, and 50 μM 7-methyl-6-thioguanosine. Quantitation of the inorganic phosphate produced in the phosphatase reaction was accomplished using the extinction coefficient of 11,200 M⁻¹ cm⁻¹ at 360 nm and pH 7.0.

Erk2 Dephosphorylation by Dual Specificity MAP Kinase Phosphatases—The MAPK phosphatases (MKPs) are dual specificity phosphatases capable of dephosphorylating both pThr and pTyr in the activation loop of MAPKs. In mammalian cells, at least ten MKPs have been identified. Previous studies suggest that some MKPs display distinct in vivo substrate preferences for the various MAPKs. For example, MKP3 has been shown to be highly specific in deactivating ERK1/2 (32–35). Using 32P-labeled ERK2/pTpY as a substrate, we previously determined the kinetic parameters of the MKP3-catalyzed dephosphorylation of ERK2/pTpY by following the production of radioactive inorganic phosphate (25). We showed that indeed ERK2/pTpY is a highly efficient substrate for MKP3 with a kcat of 0.084 ± 0.009 s⁻¹ and a Km of 22 ± 5 μM at pH 7.4 and 30 °C.

Although the radioactive assay is effective in obtaining kinetic constants, it suffers from major disadvantages of being discontinuous and labor-intensive. To establish the utility of the continuous spectrophotometric enzyme-coupled assay to the study of protein phosphatase-catalyzed MAPK dephosphorylation, we first applied this assay to analyze the dephosphorylation of ERK2/pTpY by MKP3. Figure 1 shows typical progress curves of the MKP3-catalyzed reaction at several ERK2/pTpY concentrations. As expected, there is a nice linear correlation between the final absorbance at 360 nm (which measures the total amount of inorganic phosphate released by the action of MKP3) and the amount of ERK2/pTpY present in the reaction (Figure 1, inset). The initial rates of the dephosphorylation reaction were determined from the slopes of the initial linear portion of the progress curves. Figure 2 displays the dependence of the initial rates of the MKP3-catalyzed reaction on ERK2/pTpY.
ERK2 Dephosphorylation by Protein Phosphatases

parameters for the dephosphorylation of ERK2/pT and ERK2/pY by MKP3. As can be seen from Table I, the rate of ERK2/pY dephosphorylation is slightly faster than that of ERK2/pT, and the kinetic constants for the MKP3-catalyzed dephosphorylation of ERK2/pT and ERK2/pY are comparable to those of the bisphosphorylated ERK2. Thus, both forms of the monophosphorylated ERK2 can serve as effective MKP3 substrates independently. Again, these results are similar to those obtained for the same reactions followed by radioactivity and phosphoamino acid analysis (25). Taken together, these results indicate that the continuous spectrophotometric enzyme-coupled assay is suitable for measuring the rate of inorganic phosphate production in the protein phosphatase-catalyzed MAPK dephosphorylation reaction.

We then set out to analyze the dephosphorylation of ERK2/pT and ERK2/pY by VHR and MKP5. VHR is a dual specificity phosphatase that exhibits significant sequence identity to the catalytic domains of the MKPs. Two recent papers (36, 37) suggested that ERK2/pY is a physiological substrate for VHR. Furthermore, it was shown that VHR could dephosphorylate only pTyr in ERK2/pTpY. We have confirmed that indeed VHR only catalyzed the hydrolysis of pTyr in ERK2/pTpY based on results from phosphoamino acid analysis of the product (25). Under identical conditions used for MKP3 (pH 7.4 and 30 °C), we also determined the $k_{cat}/K_m$ for the VHR-catalyzed dephosphorylation of $^{32}$P-labeled ERK2/pTpY to be $0.004 \pm 0.001 \text{ s}^{-1}$ and $3.8 \pm 0.9 \mu M$, respectively, by monitoring directly the release of $^{32}$P-labeled phosphate. Thus, the $k_{cat}/K_m$ for the VHR-catalyzed ERK2/pTpY dephosphorylation is $1.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$.

Using the continuous spectrophotometric enzyme-coupled assay, we again confirmed that VHR only dephosphorylated pTyr in ERK2/pTpY. This is based on the observation that only one equivalent of inorganic phosphate was released upon treatment of ERK2/pTpY with VHR (data not shown). Subsequent addition of HoPTP to the VHR reaction did not lead to further increase in phosphate concentration, whereas addition of PP2A did produce an additional equivalent of inorganic phosphate, most likely from the hydrolysis of the remaining ERK2/pT. Because the $K_m$ for the VHR reaction is high, we analyzed the reaction at an ERK2/pTpY concentration that was much lower than the $K_m$, to conserve the material. Under this condition, the reaction is first order with respect to [S], and the observed apparent first order rate constant is equal to $(k_{cat}/K_m)[E]$. Thus, the $k_{cat}/K_m$ value for the VHR-catalyzed dephosphorylation of ERK2/pTpY was obtained by fitting the progress curve to Equation 2 (38). As shown in Table I, the $k_{cat}/K_m$ value for the VHR-catalyzed dephosphorylation of ERK2/pTpY (2.86 $\times 10^3 \text{ M}^{-1}\text{s}^{-1}$) is only 7-fold higher than that of the hydrolysis of pNPP by VHR, an artificial chromogenic substrate, and is $>10^3$-fold lower than that for the MKP3-catalyzed ERK2/pTpY dephosphorylation ($5.60 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$). These results make it unlikely that ERK2/pTpY is a physiological substrate for VHR.

Unlike MKP3, which displays a preference for ERK2, MKP5 has been shown to associate with p38 and JNK and block the enzymatic activation of MAPKs with the selectivity of p38 = JNK ≫ ERK2 (39, 40). We found that MKP5 could remove the phosphoryl group from both pThr and pTyr in ERK2/pTpY. However, initial rate analysis suggested that the $K_m$ for the MKP5-catalyzed ERK2/pTpY dephosphorylation was $>0.73 \mu M$. Thus, the progress curve was fitted to Equation 2 and yielded a $k_{cat}/K_m$ second-order rate constant of $4.01 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$. This value is more than 100-fold lower than that of the MKP3-catalyzed ERK2/pTpY dephosphorylation and is consistent with the previous findings that MKP3 specifically down-regulates ERK2 pathways, whereas MKP5 may selectively de-activate p38 and JNK signaling.

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**Fig. 1. The time courses of ERK2/pTpY dephosphorylation by MKP3.** All experiments were performed at 25 °C and pH 7.0 in 50 mM MOPS, 100 mM NaCl, 0.1 mM EDTA. The reaction was initiated by addition of 0.051 μM MKP3. The inorganic phosphate released from ERK2/pTpY during the MKP3-catalyzed dephosphorylation was determined continuously by the coupled enzyme assay (see “Experimental Procedures”). The ERK2/pTpY concentrations were: curve 1, 0.24 μM; curve 2, 0.30 μM; curve 3, 0.36 μM; and curve 4, 0.45 μM.

**Fig. 2. Dependence of the initial velocity ($A_{meas}$/min) on ERK2/pTpY concentration for the MKP3-catalyzed reaction.** All experiments were performed at 25 °C and pH 7.0 in 50 mM MOPS, 100 mM NaCl, 0.1 mM EDTA. 0.051 μM MKP3 was used to initiate the reaction. The inorganic phosphate released from ERK2/pTpY during the MKP3-catalyzed dephosphorylation was determined continuously by the coupled enzyme assay (see “Experimental Procedures”). The data were fitted to Equation 1 to obtain the $k_m$ and $k_{cat}$ values.
ERK2 Dephosphorylation by Protein Tyrosine Phosphatases—In addition to MKPs, biochemical (41–43) and genetic (44, 45) studies indicate that distinct tyrosine-specific PTPs are involved in ERK2 inactivation. For example, PTP1B and CD45 have been shown to be capable of inactivating the fully activated ERK2 (3, 46–49). Recent evidence suggests that the hematopoietic PTP, HePTP, and its brain-specific homologs, STEP and PTP-SL, can associate with ERK2 through the N-terminal non-catalytic domain and inactivate the ERK2 kinase by dephosphorylation of pTyr in the TEY activation loop (50–53).

Using the continuous spectrophotometric assay, we examined the purified and phosphorylated ERK2 as a substrate of HePTP, PTP1B, and CD45. By fitting the initial rates versus [ERK2/pTpY] data to the Michaelis-Menten equation (Fig. 3), the \( k_{cat} \) and \( K_m \) for the HePTP-catalyzed pTyr hydrolysis of ERK2/pTpY were determined to be 1.02 ± 0.02 s\(^{-1}\) and 0.46 ± 0.03 \( \mu \)M, respectively, at pH 7.0 and 25 °C (Table II). The \( k_{cat}/K_m \) value for the HePTP-catalyzed ERK2 dephosphorylation was 2.2 \( \times 10^5 \) M\(^{-1}\) s\(^{-1}\), which is similar to that of the MKP3-catalyzed ERK2/pTpY dephosphorylation, indicating that ERK2/pTpY is also a highly efficient substrate for HePTP. Interestingly, the kinetic constants, \( k_{cat} \) and \( k_{cat}/K_m \), for the HePTP-catalyzed dephosphorylation of the monophosphorylated ERK2/pT were only slightly lower than those for the bisphosphorylated ERK2/pTpY (Table II). These results suggest that both ERK2/pT and ERK2/pTpY are efficient substrates for HePTP and that dephosphorylation of the pTyr residue by HePTP does not require the presence of pThr in the ERK2 substrate.

Neither PTP1B nor CD45 could effectively dephosphorylate ERK2/pTpY under the same conditions used for HePTP. This is striking because the catalytic activities of PTP1B and CD45 are higher than that of HePTP using pNPP as a substrate (Table II). The \( K_m \) values for the PTP1B and CD45-catalyzed ERK2/pTpY dephosphorylation reaction were too high; therefore, only \( k_{cat}/K_m \) values could be obtained. A comparison of the \( k_{cat}/K_m \) values for the PTP1B-catalyzed dephosphorylation of ERK2/pTpY and ERK2/pT indicates that pThr-183 does not have a significant impact on pTyr-185 dephosphorylation. As summarized in Table II, the \( k_{cat}/K_m \) values for the PTP1B- and CD45-catalyzed ERK2/pTpY dephosphorylation are 340- and 150-fold lower than that of the HePTP-catalyzed reaction. In fact, the \( k_{cat}/K_m \) values for the PTP1B- and CD45-catalyzed ERK2/pTpY dephosphorylation are only 1.5- and 2.9-fold higher than those for the pNPP reaction, respectively. Thus, unlike results from studies with phosphopeptides, which suggest that PTPs only exhibit moderate sequence specificity (54), our results with a physiological protein substrate, ERK2/pTpY, demonstrate that PTPs possess extremely high substrate specificity.

ERK2 Dephosphorylation by Serine/Threonine Protein Phosphatases—Among the Ser/Thr protein phosphatases, PP2A has been implicated in ERK1/2 inactivation (3, 42, 46–49, 55, 56). In contrast, PP2C has been suggested as a negative regulator of p38 and JNK kinases involved in stress responses (57–61). However, it is not clear whether PP2C or other Ser/Thr protein phosphatases are also responsible for ERK2 inactivation.

We examined the ability of all major classes of Ser/Thr protein phosphatases to dephosphorylate both ERK2/pTpY and ERK2/pT. As shown in Table III, the kinetic parameters for the hydrolysis of the commonly used phosphatase substrate pNPP by PP1, PP2A, PP2B, PP2C, and the bacteriophage λ protein phosphatase (λPP) are very similar. This indicates that these phosphatases possess similar intrinsic catalytic activity. With ERK2/pTpY as a substrate, initial rate analysis indicated that the \( K_m \) for the PP2A-catalyzed reaction was greater than 20 \( \mu \)M. Thus, we were only able to obtain a \( k_{cat}/K_m \) value of 5.55 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\) for the PP2A-catalyzed dephosphorylation of pThr in ERK2/pTpY (Table III). Interestingly, when the monophosphorylated ERK2/pT was used as a substrate, the \( k_{cat}/K_m \) value for the PP2A-catalyzed dephosphorylation was 1.21 \( \times 10^6 \) M\(^{-1}\) s\(^{-1}\), which is 22-fold higher than that for ERK2/pTpY (Fig. 4 and Table III).

There is strong evidence from genetic analyses that the PP2C-like protein phosphatases likely negatively regulate the MAPK pathways that are activated in response to osmotic and

### Table I

| Enzyme | Substrate | \( k_{cat} \) | \( K_m \) | \( k_{cat}/K_m \) |
|--------|-----------|---------------|-----------|------------------|
| MKP3   | ERK2/pTpY | 0.140 ± 0.039 | 0.025 ± 0.005 | (5.60 ± 1.55) \( \times 10^6 \) |
| MKP3   | ERK2/pT  | 0.099 ± 0.002 | 0.037 ± 0.005 | (2.68 ± 0.37) \( \times 10^6 \) |
| MKP3   | ERK2/pY  | 0.141 ± 0.005 | 0.026 ± 0.006 | (5.42 ± 1.38) \( \times 10^6 \) |
| MKP3   | pNPP     | 0.020 ± 0.002 | (9.52 ± 0.45) \( \times 10^3 \) | 2.10 ± 0.01 |
| MKP5   | ERK2/pTpY | 1.06 ± 0.04  | >>>0.73     | (4.01 ± 0.04) \( \times 10^4 \) |
| MKP5   | pNPP     |               | (9.70 ± 1.06) \( \times 10^3 \) | 109 ± 12 |
| VHR    | ERK2/pTpY | 2.25 ± 0.09  | (5.68 ± 0.59) \( \times 10^3 \) | (0.396 ± 0.041) \( \times 10^3 \) |
All experiments were performed at 25°C, 100 mM NaCl, and 0.1 mM EDTA. The reaction was initiated by addition of pTpY and ERK2/pT (Table III). Unlike PP2A, the hydrolysis of ERK2/pTpY by PP2Ca is favored by 2-fold over ERK2/pT. Our data showed that, although PP2Ca can dephosphorylate both ERK2/pTpY and ERK2/pT as efficiently as the PP2A-catalyzed ERK2/pTpY dephosphorylation, the k_cat/K_m values are still 22- to 45-fold lower than that of the PP2A-catalyzed ERK2/pT hydrolysis. It will be very informative to measure and compare the kinetic parameters for the PP2C-catalyzed dephosphorylation of p38 and/or JNK with that of ERK2 dephosphorylation in a future study.

No measurable phosphatase activity was observed for PP1, PP2B, or aPP with either ERK2/pTpY or ERK2/pT as a substrate. This was also true even when a large excess (e.g., 10× more than the amount of PP2A used) of PP1, PP2B, or aPP was added to the reactions. In a previous study (63), it was shown that incubation of partially purified ERK2 with PP2A resulted in a time-dependent inactivation of the kinase activity, whereas PP1 has no effect. Collectively, these results suggest that only PP2A and PP2Ca are capable of inactivating ERK2. However, the catalytic efficiencies for the PP2A- and PP2Ca-catalyzed dephosphorylation of ERK2/pTpY are 40- to 100-fold lower than those of HePTP and MKP3. In contrast, the dephosphorylation of ERK2/pT by PP2A is catalyzed 22-fold more efficiently, with a k_cat/K_m value close to those observed for the HePTP- and MKP3-catalyzed reactions. Thus, the preferred substrate for PP2A is the monophosphorylated ERK2/pT.

**FIG. 4. Dephosphorylation of ERK2/pTpY and ERK2/pT by PP2A.** All experiments were performed at 25°C in 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA. The reaction was initiated by addition of 0.004 μM PP2A to the reaction. The inorganic phosphate released from ERK2/pTpY during the PP2A-catalyzed dephosphorylation was determined continuously by the coupled enzyme assay (see “Experimental Procedures”). The concentration for ERK2/pTpY (curve 1) and ERK2/pT (curve 2) was 0.48 μM.

heat shock (57–61). We were able to determine both the k_cat and K_m for the PP2Ca-catalyzed dephosphorylation of ERK2/pTpY and ERK2/pT (Table III). Unlike PP2A, the hydrolysis of ERK2/pTpY by PP2Ca is favored by 2-fold over ERK2/pT. Our data showed that, although PP2Ca can dephosphorylate both ERK2/pTpY and ERK2/pT as efficiently as the PP2A-catalyzed ERK2/pTpY dephosphorylation, the k_cat/K_m values are still 22- to 45-fold lower than that of the PP2A-catalyzed ERK2/pT hydrolysis. It will be very informative to measure and compare the kinetic parameters for the PP2C-catalyzed dephosphorylation of p38 and/or JNK with that of ERK2 dephosphorylation in a future study.

No measurable phosphatase activity was observed for PP1, PP2B, or aPP with either ERK2/pTpY or ERK2/pT as a substrate. This was also true even when a large excess (e.g., 10× more than the amount of PP2A used) of PP1, PP2B, or aPP was added to the reactions. In a previous study (63), it was shown that incubation of partially purified ERK2 with PP2A resulted in a time-dependent inactivation of the kinase activity, whereas PP1 has no effect. Collectively, these results suggest that only PP2A and PP2Ca are capable of inactivating ERK2. However, the catalytic efficiencies for the PP2A- and PP2Ca-catalyzed dephosphorylation of ERK2/pTpY are 40- to 100-fold lower than those of HePTP and MKP3. In contrast, the dephosphorylation of ERK2/pT by PP2A is catalyzed 22-fold more efficiently, with a k_cat/K_m value close to those observed for the HePTP- and MKP3-catalyzed reactions. Thus, the preferred substrate for PP2A is the monophosphorylated ERK2/pT.

**TABLE II**

| Enzyme | Substrate   | k_cat | K_m  | k_cat/K_m |
|--------|-------------|-------|------|----------|
| HePTP  | ERK2/pTpY   | 1.02  | 0.46 | (2.22 ± 0.12) × 10^6 |
| HePTP  | ERK2/pY     | 0.774 | 0.58 | (1.33 ± 0.19) × 10^6 |
| HePTP  | pNPP        | 3.63  | (4.66 ± 0.10) × 10^5 |
| PTP1B  | ERK2/pTpY   | 8.83  | 2.00 | (4.41 ± 0.22) × 10^3 |
| CD45   | ERK2/pTpY   | 25.5  | (4.93 ± 0.38) × 10^3 |
Protein Phosphatases Possess Extraordinarily High Intrinsic Substrate Specificity toward Physiologically Relevant Protein Substrates—From the results presented above, it is clear that only HePTP, MKP3, and PP2A have the level of specificity and activity to support them being physiologically relevant regulators of ERK2 kinase activity. This conclusion is borne out from a comparison of the $k_{\text{cat}}/K_m$ values for ERK2 dephosphorylation catalyzed by various phosphatases (Tables I–III). The second order rate constant $k_{\text{cat}}/K_m$ (also called substrate specificity constant) is a physiologically relevant parameter for the reaction of free enzyme with free substrate and reflects both binding affinity and catalytic efficiency. The $k_{\text{cat}}/K_m$ values determined at pH 7 and 25 °C for the HePTP-catalyzed dephosphorylation of ERK2/pTpY and ERK2/pY; the MKP3-catalyzed dephosphorylation of ERK2/pTpY, ERK2/pT, and ERK2pY; and the PP2A-catalyzed dephosphorylation of ERK2/pT are greater than $10^6 \text{ M}^{-1}\text{s}^{-1}$ and represent the largest values measured for a protein phosphatase reaction. In contrast, the $k_{\text{cat}}/K_m$ values for ERK2 dephosphorylation catalyzed by PTP1B, CD45, VHR, MKP5, and PP2Ca were at least two orders of magnitude lower, whereas PP1, PP2B, and the λPP were unable to dephosphorylate ERK2.

The results collectively demonstrated that protein phosphatases display exquisite substrate specificity toward this physiologically relevant protein substrate, the phosphorylated ERK2. This is in stark contrast to results from studies with low molecular weight artificial substrates or with substrates immunoprecipitated from cell lysates assayed at a high phosphate concentration. This specificity is evident even though the catalytic domains among the PTPs, the MKPs, and the PPP family Ser/Thr phosphatases display greater than 50% amino acid sequence identity. PTP1B and CD45 are more efficient than HePTP in hydrolyzing pNPP- and pTyr-containing peptides (refs. 64 and 65 and Table II), but they do not react with the phosphorylated ERK2 efficiently. The results suggest that substrate recognition by protein phosphatases may involve extensive protein-protein contacts in addition to the interactions that engage the phosphoamino acid residue. Indeed, both HePTP (52) and MKP3 (20) contain an ERK2 kinase interaction (KIM) motif that mediates phosphatase-substrate association using regions of ERK2 distant from the phosphorylation sites. Thus, protein-protein interactions between the phosphatase and its substrate involving both the enzymatic active site and adjacent or remote noncatalytic sites may be responsible for the observed high substrate specificity. Interestingly, the A and C subunits of PP2A do not appear to have the canonical KIM sequence, which may explain why PP2A has a high $K_m$ value for ERK2.

Potential Biological Significance of ERK2 Inactivation by Multiple Phosphatases—There are thousands of potential protein phosphatase substrates in the cell. Many of the phosphoproteins contain multiple phosphorylation sites. However, it remains largely unknown as how many phosphatases are involved in dephosphorylation of a single substrate. Conversely, it is also unclear how many substrates can be efficiently processed by one phosphatase. Our results highlight the importance of carrying out detailed and stringent kinetic analysis of substrate dephosphorylation to identify and validate physiological substrates of protein phosphatases.

Our kinetic analysis shows that the tyrosine-specific HePTP, the Ser/Thr-specific PP2A, and the dual-specific MKP3 are all highly specific for ERK2 dephosphorylation. This is interesting, because it is well known that both the magnitude and duration of ERK2 activation are important in determining cell fate (66). Thus, the level of ERK2 phosphorylation, and therefore the strength and duration of the ERK2 signal transmitted, are balanced by the opposing action of MEK1 and multiple protein phosphatases. The fact that ERK2 activation is catalyzed by a single kinase and its inactivation could be carried out by multiple phosphatases implies that diverse signals could be integrated at the phosphatase level in determining the cellular responses to external stimuli.

It is well established that full activation of MAPKs require dual phosphorylation of the Thr and Tyr residues in the activation loop. However, in much of the literature investigating MAPK signaling, it is widely assumed that the monophosphorylated forms are inactive. Thus, the significance for the need of multiple phosphatases in regulating MAPK activity is not clear. It is also unclear whether the monophosphorylated ERK2s have any distinct biological functions even though both ERK2/pT and ERK2/pY can be detected in living cells in addition to the unphosphorylated ERK2 and the bisphosphorylated ERK2/pTpY (62, 67). We have recently shown that a single phosphorylation in the activation loop of ERK2 produces an intermediate activity state (23). Thus, the catalytic efficiencies of the monophosphorylated 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 bisphosphorylated ERK2/pTpY. This raises the possibility that the monophosphorylated ERK2s may have distinct biological roles in vivo.

MEK1 phosphorylates ERK2 by a two-collision, distributive mechanism, which may be important for the fidelity and sensitivity of ERK2 signaling (9, 10). The specificity of ERK2 activation should be greater under a distributive mechanism than under a processive mechanism, because activation depends upon two independent MEK1 binding events rather than one. The MKP3-catalyzed efficient removal of phosphate group from both pThr and pTyr of the activated ERK2 may be important for prolonged inactivation and efficient repriming of the ERK signaling cycle. On the other hand, the coordinated action of HePTP and PP2A on ERK2/pTpY would generate monophosphorylated ERK2 species, which would allow MEK1 to activate ERK2 more rapidly under certain conditions. Thus, the use of both single specificity (Ser/Thr- or Tyr-specific) and dual specificity phosphatases for ERK2 inactivation may have an impact on the time course, the threshold for re-activation, and the efficiency of regulation of the ERK2 pathways. Furthermore, 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 Order of ERK2/pTpY Dephosphorylation by HePTP and PP2A—As discussed above, the cell’s use of single-specificity phosphatases may reflect an additional level of regulation for MAPK signaling not yet fully appreciated, however, a detailed understanding of the mechanism by these phosphatases is not available. Previous studies suggest that the dephosphorylation of pThr-183 and pTyr-185 of ERK2 are catalyzed by distinct protein phosphatases in certain cell types. For example, it was shown, based on biochemical analysis of cell extracts using phosphatase inhibitors okadaic acid and vanadate, that pThr-183 dephosphorylation is rate-limiting and pTyr-185 dephosphorylation is inhibited by the phosphorylation of Thr-183 in PC12 cells (42). However, in a separate study that involved microinjection of ERK2/pTpY to oocytes, Sohaskey and Ferrell (43) showed that ERK2 pThr-183 dephosphorylation is contingent upon pTyr-185 dephosphorylation, i.e. the efficiency of Thr-183 dephosphorylation is increased by prior pTyr-185 dephosphorylation.
Our direct kinetic measurements showed that the rate of pTyr-185 hydrolysis catalyzed by HePTP (and possibly its brain-specific homologs STEP/PTP-SE) is independent of the presence of pThr-183, indicating that the ERK2-specific PTP can work on both ERK2/pTyr and ERK2/pY. Thus, the action of HePTP in ERK2 inactivation does not depend on the activity of serine/threonine phosphatases such as PP2A. By contrast, our data clearly demonstrated that the dephosphorylation of pThr-183 in ERK2/pY. This suggests that the presence of pTyr-185 is inhibitory to the PP2A-catalyzed pThr-183 dephosphorylation. Although there may be different mechanisms for ERK2 dephosphorylation in different cell types, our results are consistent with those of Sohasky and Ferrell (43) and suggest that the HePTP family of PTPs may be the initial phosphatases acting on ERK2. Continued examination of the role of HePTP and PP2A and how they act together with MKP3 to regulate the ERK2 pathway should provide further insights into the regulation of MAPK pathways by protein phosphatases.

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