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New Insights into the Catalytic Activation of the MAPK Phosphatase PAC-1 Induced by its Substrate MAPK ERK2 Binding

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PAC-1 is an inducible, nuclear-specific, dual-specificity mitogen-activated protein (MAP) kinase phosphatase that has been shown recently to be a transcription target of the human tumor-suppressor protein p53 in signaling apoptosis and growth suppression. However, its substrate specificity and regulation of catalytic activity thus far remain elusive. Here, we report *in vitro* characterization of PAC-1 phosphatase activity with three distinct MAP kinase subfamilies. We show that the recombinant PAC-1 exists in a virtually inactive state when alone *in vitro*, and dephosphorylates extracellular signal-regulated kinase 2 (ERK2) but not p38α or c-Jun NH_2-terminal kinase 2 (JNK2). ERK2 dephosphorylation by PAC-1 requires association of its amino-terminal domain with ERK2 that results in catalytic activation of the phosphatase. p38α also interacts with but does not activate PAC-1, whereas JNK2 does not bind to or cause catalytic activation by PAC-1. Moreover, our structure-based analysis reveals that individual mutation of the conserved Arg294 and Arg295 that likely comprise the phosphothreonine-binding pocket in PAC-1 to either alanine or lysine results in a nearly complete loss of its phosphatase activity even in the presence of ERK2. These results suggest that Arg294 and Arg295 play an important role in PAC-1 catalytic activation induced by ERK2 binding.

Keywords: mitogen-activated protein kinase; extracellular signal-regulated kinase; c-Jun NH_2-terminal kinase; MAPK phosphatase; PAC-1

Introduction

Mitogen-activated protein kinases (MAPKs) are key components of the Ras-mediated MAPK signaling pathways that relay signals from the cell-surface receptors to the nucleus to activate gene transcription in response to extracellular stimulation by growth factors or environmental stresses.[1–3] MAPKs are grouped into three major classes on the basis of their preferential activation by extracellular stimuli: the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38.[4,5] The biological importance of MAPKs is underscored by tight control of their activity *via* phosphorylation of two different amino acid residues, i.e. the threonine and tyrosine in a TXY motif in the activation loop, where *X* is glutamate, proline or glycine, respectively, in ERK, JNK or p38. Phosphorylation of both threonine and tyrosine residues is required to maintain high activity of an MAPK.[6] Conversely, dephosphorylation of MAPKs rapidly inactivates their activity.

More than ten mammalian dual-specificity MAPK phosphatases (MKPs) have been identified and shown to be capable of dephosphorylating both phosphothreonine (pT) and phosphotyrosine (pY) in the activation loop, thus inactivating

Abbreviations used: ERK, extracellular signal-regulated kinase; GST, glutathione-*S*-transferase; JNK, c-Jun NH_2-terminal kinase; KIM, kinase-interaction motif; MAP, mitogen- activated protein; MAPK, mitogen-activated protein kinase; MKP, MAPK phosphatase; *p*-NPP, *p*-nitrophenyl phosphate; pT, phosphothreonine; pY, phosphotyrosine.

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MAPKs.[7,8] These dual specificity MKPs all consist of an amino-terminal kinase-binding domain and a carboxy-terminal phosphatase domain. The highly conserved dual-specificity phosphatase domain contains the tyrosine-specific phosphatase signature sequence HCXXXXXR at the active site, where cysteine acts as the enzymatic nucleophile and arginine coordinates the phosphate group of pY or pT in the dephosphorylation reaction, and X is any amino acid. The kinase-binding domain of an MKP recognizes selective MAPKs *via* its conserved kinase-interaction motif (KIM) containing the XXRRXXKXXLXV sequence, thus contributing directly to substrate specificity of the MKP.[9–14] More strikingly, this direct enzyme–substrate interaction causes catalytic activation of many MKPs[9,11,15,16] that otherwise exhibit very low phosphatase activity in the absence of substrates. Structural and biochemical analyses further suggest that the tightly controlled enzymatic activity of these MKPs requires structural rearrangement of the active site residues in a substrate-induced activation mechanism,[11,17,18] which is different from the auto-inhibition mechanism seen commonly in other protein phosphatases such as the SHP-2 tyrosine phosphatase.[19] However, the detailed structural basis of substrate specificity and MAPK-induced catalytic activation of MKPs is yet to be determined.

Biological function of MKPs is dependent also on their subcellular localization and transcriptional regulation. For example, MKP-3 (Pyst1),[20,21] Pyst2,[21] MKP-4,[22] MKP-5,[23] MKP-6, MKP-7,[24] and M3/6,[25] which are present predominantly in the cytosol, play an important role in regulating the functions of MAPKs in signal transduction, receptor tyrosine kinase endocytosis and stress-induced apoptosis. On the other hand, MKP-1 (Cl100/3CH134),[26,27] MKP-2,[28,29] PAC-1,[30,31] and B23 (VH3),[32,33] which are exclusively in the nucleus and encoded by immediate early genes induced by the activated MAPKs upon nuclear entrance. Indeed, it has been shown that the nuclear-specific MKPs are induced rapidly by the extracellular stimuli that also induce activation of MAPKs, suggesting that these inducible MKPs function as negative regulators in a feedback control mechanism that controls the functions of MAPKs in gene transcription.

Notably, the nuclear-specific PAC-1 has been shown recently to be a transcription target of the human tumor suppressor protein p53, and acts as a critical downstream effector of p53 in initiating apoptosis to suppress tumorigenesis.[34] PAC-1, which is expressed predominantly in hematopoietic cells, is absent from quiescent cells, and its expression is inducible by oxidative stress in a p53-dependent manner to act as a cell-death mediator in the cellular response to oxidative damage or nutritional stress.[30,34] PAC-1 was first discovered as an MKP due to its function in inactivating ERKs in T-cell activation;[30,31] however, knowledge of substrate specificity and regulation of PAC-1 is limited.

In this study, we have examined the substrate specificity of PAC-1 *in vitro* using recombinant and purified substrates of three distinct MAPK subfamilies. Our study shows that the recombinant PAC-1 is virtually inactive when alone, and can dephosphorylate ERK2 but not p38α or JNK2 *in vitro*. ERK2 dephosphorylation by PAC-1 requires direct association between the amino-terminal domain of PAC-1 and ERK2 that results in catalytic activation of the MAPK phosphatase. On the contrary, PAC-1 forms a stable complex with p38α, but it does not exhibit any catalytic activation by p38α; PAC-1 does not interact with JNK2, nor does it show any catalytic activation by JNK2. Moreover, our structure-based mutagenesis further reveals that mutation of the conserved Arg294 or Arg295 in the phosphothreonine-binding pocket to either alanine or lysine, which does not affect PAC-1/ERK2 complex formation, causes a complete loss of ERK2-induced catalytic activation of the phosphatase. Our findings suggest that both Arg294 and Arg295 are likely involved in PAC-1 catalytic activation induced by ERK2 binding.

Results

PAC-1 dephosphorylates ERK2 preferentially

To study the structure and function of PAC-1, the cDNA encoding the full-length PAC-1 was cloned from a mouse spleen cDNA library. While the protein sequence of the human PAC-1 is highly homologous to that of the mouse counterpart, with about 80% identity, the level of expression of the former enzyme was extremely low in the bacterial expression system, thus preventing us from obtaining enough of the recombinant protein for structure–function studies. The recombinant mouse PAC-1 was expressed in bacterial BL21(DE3) cells as a soluble glutathione-*S*-transferase (GST) fusion protein, purified by glutathione-Sepharose affinity chromatography followed by a gel-filtration chromatography. The second purification step was used to remove GST, which likely resulted from non-specific cleavage of the GST-fusion PAC-1 by bacterial proteases upon protein expression (Supplementary Data, Figure S1A and B). The purity of the recombinant GST-PAC-1 protein obtained from this procedure was estimated to be about 90%, and the identity of GST-PAC-1 was confirmed by mass spectrometry analysis, as well as by Western blot using anti-GST antibody. We further expressed and purified three distinct MAPKs, ERK2, p38α and JNK2, using methods described in Experimental Procedures. All three recombinant MAPKs were dual-phosphorylated in the activation loop *in vitro*, confirmed by Western blot analysis using antibodies specific for the active and dual-phosphorylated forms of these three different MAPKs.

To understand the substrate specificity of PAC-1, we examined dephosphorylation of these three different MAPKs by the recombinant PAC-1 in an

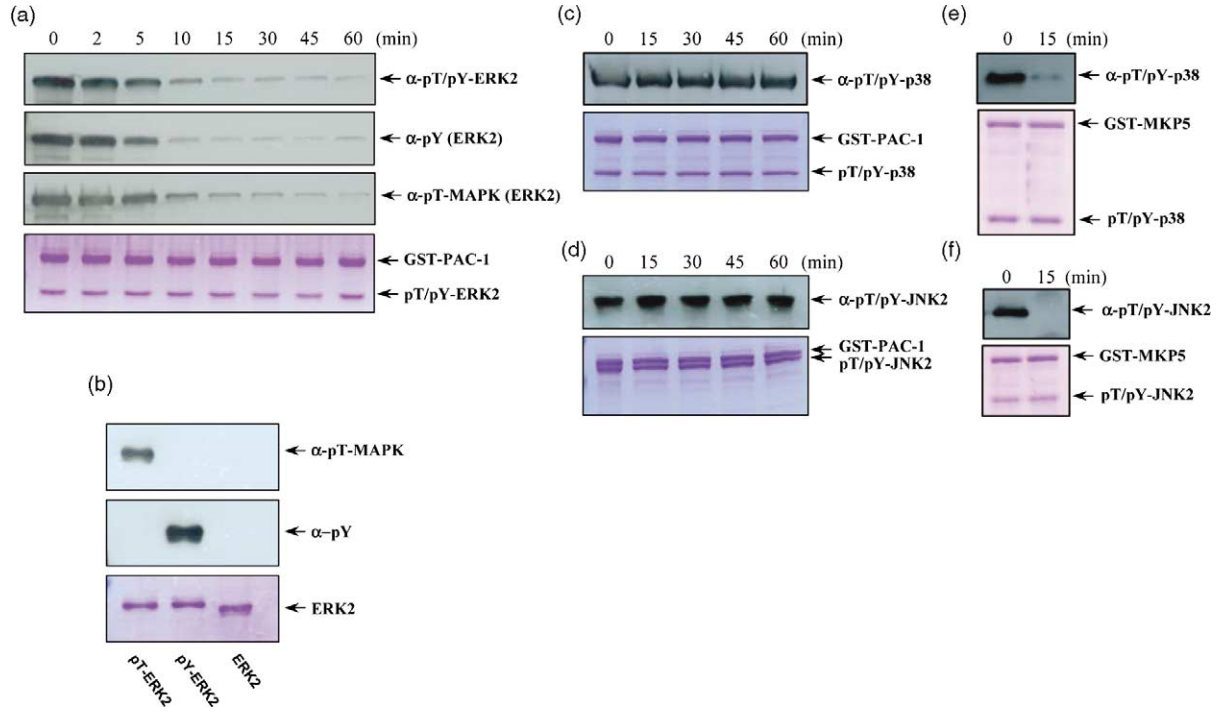

Figure 1. Dephosphorylation of MAPKs by the mouse PAC-1. (a) Western blots (upper three panels) with anti-ACTIVE ERK2, anti-phosphotyrosine and anti-phosphothreonine MAPK antibodies, showing the phosphorylation state of phosphotyrosine and phosphothreonine of the dual-phosphorylated ERK2 in a time-course of PAC-1 treatment at 37 °C. The details of the reaction conditions are described in Experimental Procedures. The lower panel of an SDS-PAGE gel stained with Coomassie brilliant blue depicts relative equal amounts of the purified GST-PAC-1 (2.5 μM) and the dual-phosphorylated ERK2 (2.5 μM) used in each time-point. (b) Western blot analysis of pT-ERK2, pY-ERK2 and ERK2 by anti-phosphothreonine-MAPK (upper panel) and anti-phosphotyrosine (middle panel) antibodies. An SDS-PAGE gel (lower panel) shows relatively equal amounts of ERK2 proteins were used in these experiments (2.5 μM). (c) and (d) Western blots (upper panels) with anti-ACTIVE (pT/pY) p38α and anti-ACTIVE (pT/pY) JNK2 antibodies, illustrating the phosphorylation state of p38α and JNK2, respectively, during the GST-PAC-1 treatment. (e) and (f) Western blotting analyses of p38α and JNK2 dephosphorylation by MKP-5.

equal molar ratio (2.5 μM) in a time-dependent manner (Figure 1). While PAC-1 exhibits almost no detectable phosphatase activity towards the dual-phosphorylated p38α or JNK2 (Figure 1(c) and (d)), it can dephosphorylate ERK2 rapidly; the dual-phosphorylated ERK2 is ~80% dephosphorylated after treatment with PAC-1 for 10 min at 37 °C, and becomes nearly completely dephosphorylated after 60 min (Figure 1(a), top panel). Western blot analysis of the ERK2 dephosphorylation reaction by using specific antibodies against phosphotyrosine, and threonine-phosphorylated MAPK (Figure 1(b)) further demonstrates that PAC-1 can dephosphorylate both phosphorylated amino acid residues in ERK2 effectively, and dephosphorylation of phosphotyrosine is slightly faster than that of phosphothreonine (Figure 1(a), two lower panels). This observation is consistent with ERK2 dephosphorylation by the cytoplasmic MKP-3.[16] To rule out the possibility that the lack of PAC-1 phosphatase activity towards p38α and JNK2 was due to incorrect phosphorylation of the MAPKs, we treated the dual-phosphorylated p38α and JNK2 by MKP-5, which has been shown to dephosphorylate p38α and JNK2 in preference to ERK2.[23] As shown in Figure 2(e) and (f), both MAPKs can be dephosphorylated rapidly by MKP-5, thus confirming that p38α and JNK2 were likely phosphorylated correctly. Taken together, these results demonstrate that PAC-1 has a highly selective, dual-specificity phosphatase activity towards ERK2 and is not active for dephosphorylation of p38α or JNK2.

PAC-1 binds to ERK2 and p38α but not to JNK2

To determine whether phosphatase activity of PAC-1 towards different MAPKs is dependent on its ability to interact with MAPKs, we performed PAC-1/MAPK binding study in a GST pull-down assay. The purified, dual-phosphorylated His-tagged MAPKs were incubated with GST-PAC-1 that was immobilized onto glutathione-Sepharose beads. The beads were washed extensively with the binding buffer to remove any non-specific binding proteins, and the proteins bound to the beads were separated by SDS-PAGE and analyzed by Western blot using anti-GST and anti-His-tag antibodies. Whereas His-tagged ERK2 showed no any detectable non-specific binding to GST, it was brought down by GST-PAC-1 (Figure 2(A), lane 1). Because of effective dephosphorylation by PAC-1 (Figure 1(a)), ERK2 treated with PAC-1 is likely

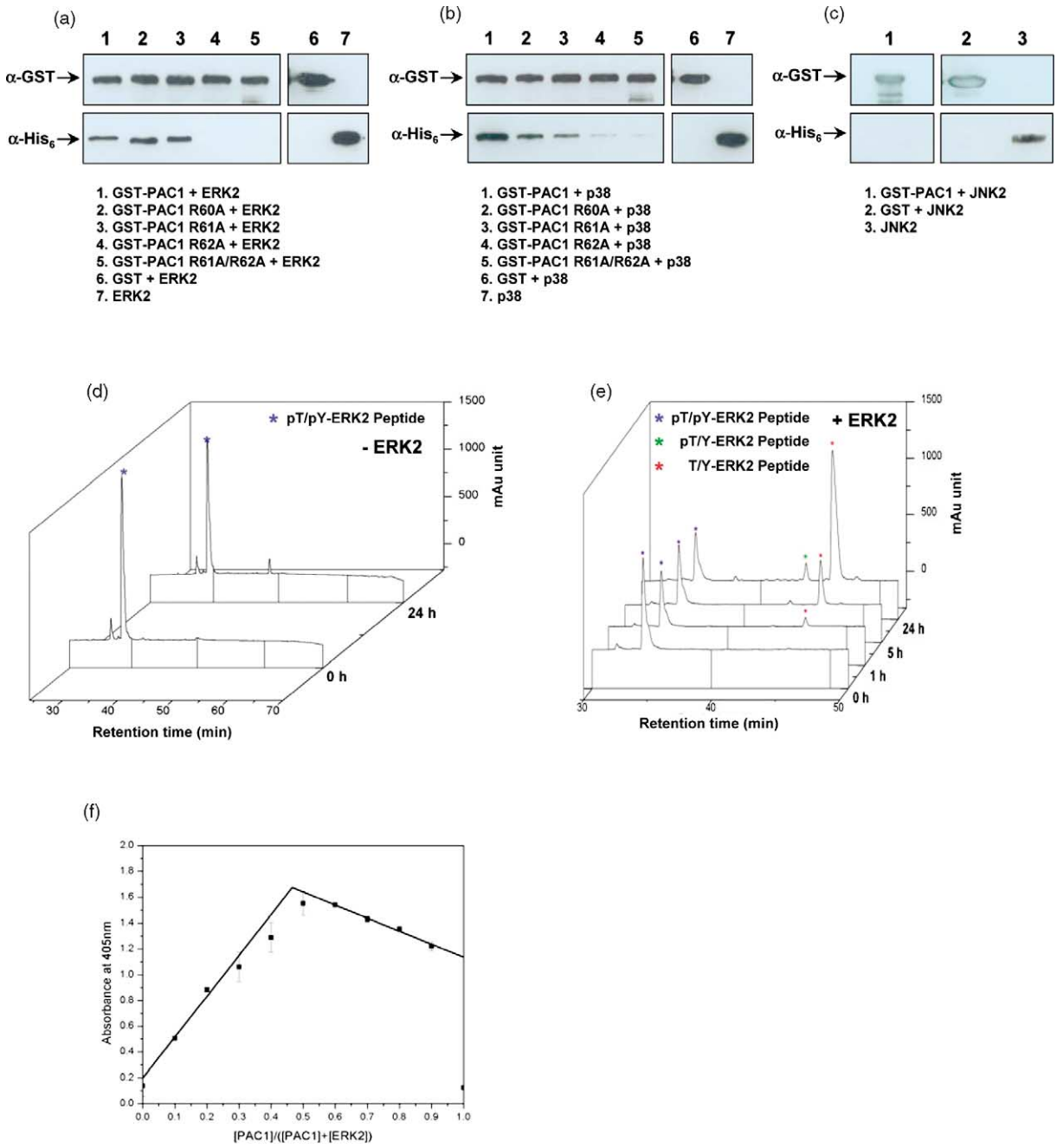

Figure 2. Direct binding between PAC-1 and MAPKs. Wild-type GST-PAC-1, mutants or GST immobilized to glutathione-Sepharose beads were incubated with the purified, His_6-tagged dual-phosphorylated (a) ERK2; (b) p38α; and (c) JNK2, and the beads were washed extensively to remove any non-specific protein binding. The direct MAPK/PAC-1 binding was visualized by Western blot with anti-His_6-tag antibody (lower panel). Relatively equal amounts of GST-PAC-1 (5 μM) and MAPK (10 μM) was used in each experiment, as shown by Western blot using anti-GST antibody (upper panel). In the control experiments, ~30 μM ERK2 (lane 7 in (a)) and p38 (lane 7 in (b)) and ~10 μM JNK2 (lane 3 in (c)) were used. (d) and (e) Dephosphorylation of a dual-phosphorylated ERK2 peptide (DHTGFL-pT-E-pY-VATR, residues 184–196 in ERK2) by PAC-1 in the absence and in the presence of ERK2, as illustrated by reversed-phase HPLC analysis. The identity of the dephosphorylated products of the ERK2 peptide was confirmed by mass spectrometric analysis. The concentration of peptide was kept at ~1 mM, and reaction was conducted in the phosphate assay buffer condition as described in Experimental Procedures. (f) The Job plot of PAC-1 activation by ERK2 measured by pNPP hydrolysis, illustrating a 1:1 stoichiometry of the PAC-1/ERK2 complex.

the dephosphorylated form, which migrates in the SDS-PAGE gel slightly faster than its phosphorylated form (see below, Figure 3(b)). This confirms that PAC-1/ERK2 binding is not dependent upon phosphorylation of the latter protein, and PAC-1 still forms a complex with dephosphorylated ERK2. PAC-1 binds also to p38α (Figure 2(b), lane 1), although the binding does not result in

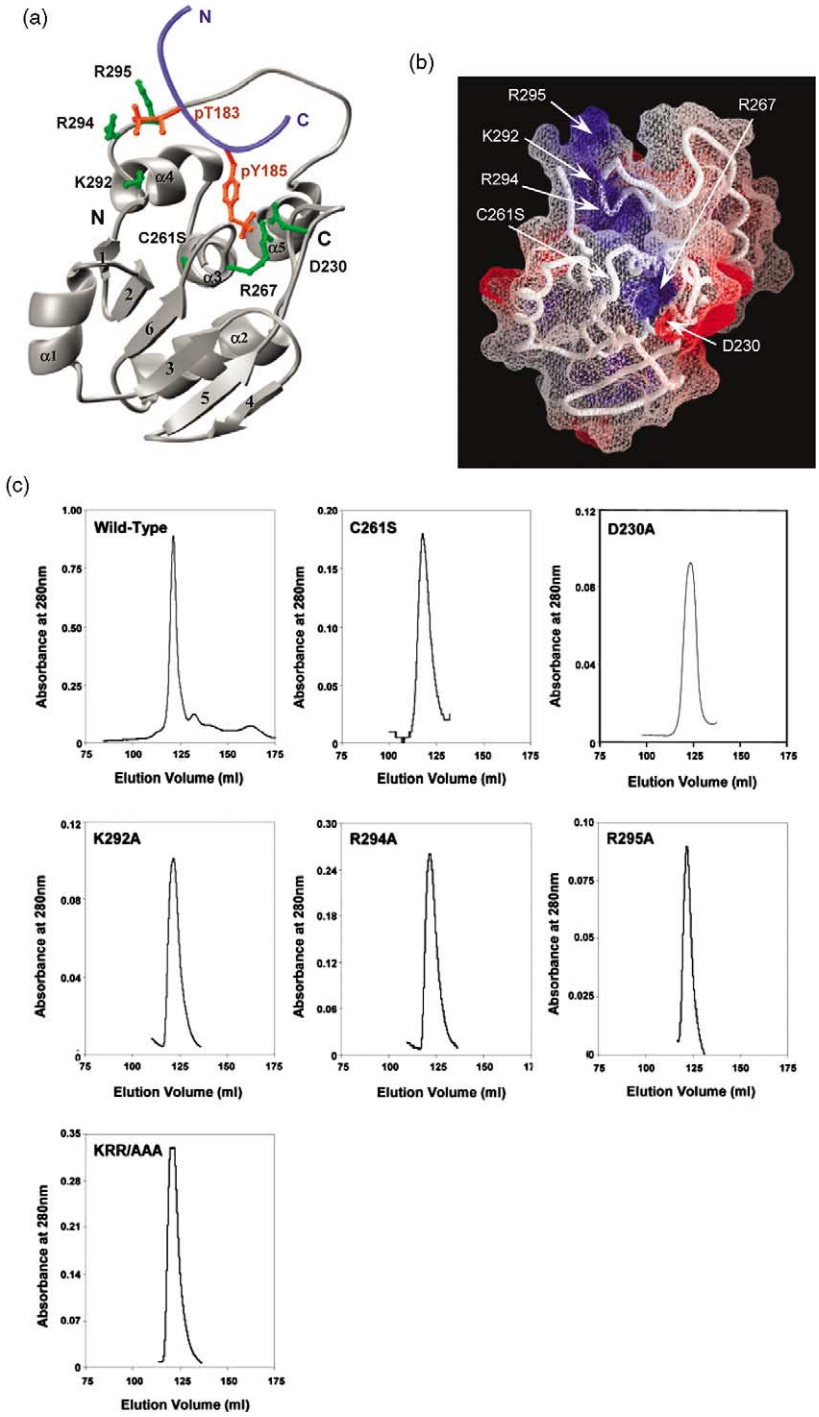

Figure 3. Effect of mutations of the active site residues on the overall conformation of PAC-1. (a) Ribbon diagram of the model structure of the phosphatase domain of the PAC-1 catalytic domain in complex with an ERK2 peptide, illustrating location of key amino acid residues at the phosphotyrosine and phosphothreonine-binding sites. The model was generated on the basis of the free form PAC-1 structure and the putative interactions of phosphotyrosine with R267 and of phosphothreonine with R294 and R295. (b) Surface representation of the electrostatic potentials of the PAC-1 phosphatase domain. Positively and negatively charged amino acid residues are color-coded blue and red, respectively. (c) Gel-filtration chromatography profiles of the wild-type PAC1 and the active site mutants.

dephosphorylation of p38α (Figure 1(b)). Moreover, a similar GST pull-down study shows that PAC-1 does not bind to JNK2 (Figure 2(c), lane 1). It is important to note that all three MAPK proteins are folded properly, as supported by their gel-filtration chromatography profiles (data not shown).

It has been shown that MKP-3 interacts selectively with ERK2 *via* its conserved kinase-interaction motif of the XXRRXXKXXLXV sequence in the amino-terminal domain.[13,35] To understand the molecular basis of PAC-1 binding to ERK2 and p38α, we mutated the corresponding residues Arg60, Arg61 and Arg62 in the KIM region in PAC-1 individually or collectively to alanine. Analysis of these mutants shows that alanine substitution of Arg60 or Arg61 had little, if any, effect on PAC-1/ERK2 (Figure 2(a), lanes 2 and 3), whereas the single R62A and the double R61A/R62A mutants resulted in a complete loss of the PAC-1/ERK2 binding (Figure 2(a), lanes 4 and 5), suggesting that Arg62 in the KIM sequence plays an essential role in PAC-1/ERK2 association. On the

other hand, all three arginine residues seem to contribute to PAC-1 binding to p38α in the order: R62 > R61 > R60 (Figure 2(b), lanes 2–4). Moreover, it appears that in addition to these three arginine residues, other region(s) of p38α may contribute to PAC-1, although the KIM arginine-mediated interactions are likely to be predominant in PAC-1/p38α interaction (Figure 2(b), lane 5). Collectively, these results suggest that PAC-1 likely employs its KIM sequence to interact with ERK2 or p38α, and that the former leads to dephosphorylation by PAC-1, and the latter does not.

Enzymatic activation of PAC-1 by ERK2

To assess whether ERK2 binding affects the enzymatic activity of PAC-1 directly, we measured the phosphatase activity on dephosphorylation of a dual-phosphorylated ERK2 peptide (DHTGFL-pT-E-pY-VATR, residues 184–196 in ERK2). The reaction products were separated by using reverse-phase HPLC and analyzed by mass spectrometry. The free PAC-1 exhibited little, if any, catalytic activity on dephosphorylation of the dual-phosphorylated pT/pY-ERK2 peptide (Figure 2(d)), and addition of ERK2 dramatically stimulated dephosphorylation of phosphotyrosine by PAC-1 followed by dephosphorylation of phosphothreonine (Figure 2(e)). PAC-1 catalytic activation by ERK2 binding is demonstrated by measurement of its enzymatic parameters towards hydrolysis of *p*-nitrophenyl phosphate (*p*-NPP) (Table 1). The Job plot analysis of PAC-1 catalytic activation induced by ERK2 binding further suggests a stoichiometry of 1:1 of the PAC-1/ERK2 complex (Figure 2(f)). Neither p38α nor JNK2 causes catalytic activation of PAC-1 as measured in *p*-NPP hydrolysis (Table 1). These results confirm that binding of the amino-terminal domain of PAC-1 to ERK2 causes catalytic activation of PAC-1, which in turn results in dephosphorylation of ERK2 by PAC-1. Taken together, the data presented here suggest that ERK2, but not JNK2, is likely a physiological substrate for PAC-1 in cell signaling. However, our *in vitro* data cannot let us decide whether p38α is a physiological substrate for PAC-1.

Mutational analysis of the enzyme active site residues of PAC-1

In our recent structural analysis of the carboxy-terminal phosphatase domain of PAC-1 with NMR,[18] we have identified a second phospho-amino acid binding pocket adjacent to the enzymatic active site, as illustrated by a model structure of the PAC-1 catalytic domain in complex with a pT/pY-ERK2 peptide (Figure 3(a)). This positively charged binding concave, which was discovered from NMR titration with phospho-amino acids and their chemical analogs and is consistent with the recently reported crystal structure of VHR/p38 phosphopeptide complex,[36] consist of three conserved, positively charged amino acid residues, Lys292, Arg294, and Arg295 (Figure 3(b)).

To assess the role of these positively charged residues in PAC-1 function, we performed site-directed mutagenesis by changing these residues individually or collectively to alanine. Since all three residues are located in a flexible loop connecting α4 and α5 in PAC-1,[18] we expected that such conserved mutations would not cause significant structural perturbations of the enzyme. This rationale is indeed supported by the behavior of the purified wild-type and mutant PAC-1 proteins in gel-filtration chromatography, in which all PAC-1 proteins showed a symmetric elution peak with nearly identical elution volume (Figure 3(c)). As shown in Figure 4(a), alanine substitution of Lys292 does not affect the overall ability of PAC-1 to dephosphorylate ERK2, although while dephosphorylation of pY by the K292A mutant was not altered, its dephosphorylation of pT appears to be slowed slightly as compared to those by the wild type PAC-1 (Figures 1(a) and 4(a)). More strikingly, both PAC-1 mutants of R294A and R295A completely abrogated the catalytic activity to dephosphorylate ERK2 (Figure 4(b) and (c)). Similarly, change of Arg294 or Arg295 to lysine also resulted in a nearly complete loss of phosphatase activity of PAC-1 in hydrolysis of *p*-NPP (Table 1). This is further confirmed by a complete loss of PAC-1 activity towards ERK2 by a triple mutant, in which all three positively charged residues, Lys292, Arg294 and Arg295, were changed to alanine (Figure 4(d)).

Table 1. Kinetic parameters of PAC-1 with *p*-NPP as a substrate

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)
PAC-1	–	N/A	–
PAC-1 + ERK2	2.13 ± 0.47	0.085 ± 0.004	39.9 ± 1.2
PAC-1 + p38	–	N/A	–
PAC-1 + JNK2	–	N/A	–
PAC-1_K292A + ERK2	2.43 ± 0.37	0.081 ± 0.003	33.3 ± 1.0
PAC-1_R294A + ERK2	–	N/A	–
PAC-1_R295A + ERK2	–	N/A	–
PAC-1_R294K + ERK2		N/A	
PAC-1_R295K + ERK2		N/A	

N/A, no activity towards *p*-NPP hydrolysis.

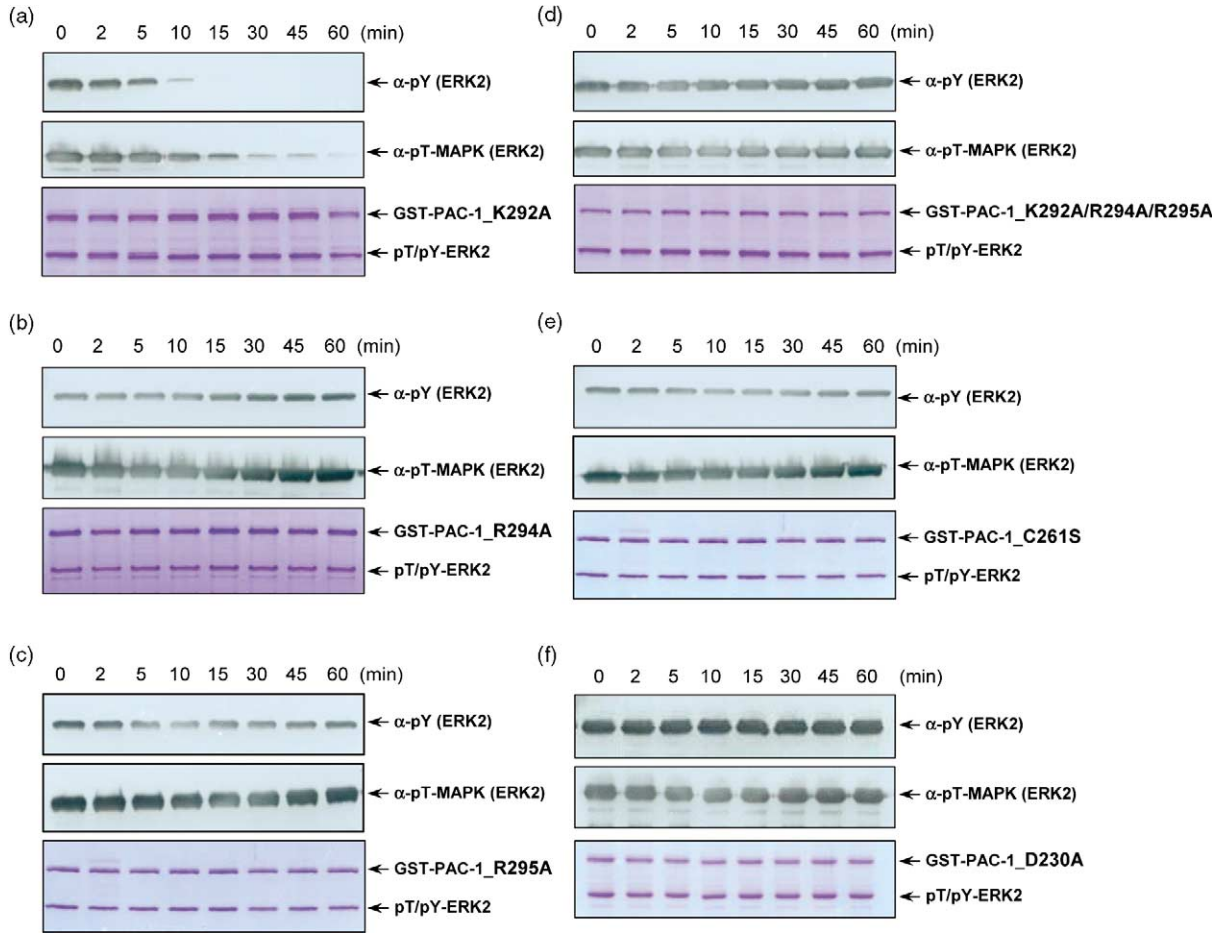

Figure 4. Effects of the active site mutations on dephosphorylation of ERK2 by PAC-1. (a)–(f) Western blots with anti-phosphotyrosine and anti-phosphothreonine MAPK antibodies (upper and middle panels) showing a time-course of dephosphorylation of ERK2 by the treatment of K292A, R294A, R295A, K292A/R294A/R295A, C261S or D230A mutants of PAC-1, respectively. The lower panels of SDS-PAGE gels stained with Coomassie brilliant blue showing relatively equal amounts of the purified GST-PAC-1 mutants (~2.5 μM) and the dual-phosphorylated ERK2 (~2.5 μM) used in each experiment.

The complete loss of the phosphatase activity of these arginine mutants is similar to that observed with a PAC-1 mutant with substitution of the enzymatic nucleophile C261 to serine (Figure 4(e)) or of the putative general acid D230 to alanine (Figure 4(f)).

A GST pull-down binding study shows that all of these PAC-1 mutants can form a stable complex with ERK2 as well as the wild-type PAC-1 (Figure 5(a)), arguing that none of these mutations at the phosphothreonine binding pocket or at the enzyme catalytic site affects PAC-1/ERK2 complex formation, which is accomplished mostly by the KIM sequence of the amino-terminal domain of PAC-1. Therefore, these results suggest that both conserved Arg294 and Arg295, which are involved in binding to phosphothreonine of the dual-phosphorylated ERK2, are essential for effective dephosphorylation of ERK2 by PAC-1.

The role of Arg294 and Arg295 in ERK2-induced enzymatic activation of PAC-1

To further understand effects of mutations of the phosphothreonine-binding residues on the catalytic activity of PAC-1, we examined ERK2-induced enzymatic activation of the PAC-1 mutants in the *p*-NPP hydrolysis assay. As expected, K292A mutant exhibited an ERK2 dose-dependent catalytic activation, almost exactly the same as that of the wild-type PAC-1 (Figure 5(b)), consistent with K292A's nearly full dual-specificity phosphatase activity in ERK2 dephosphorylation (Table 1). Both R294A and R295A mutants appeared to be completely inactive enzymes, with no response to ERK2 stimulation, in a manner similar to that of the inactive active-site mutant C261S.

Because PAC-1 effectively dephosphorylates ERK2, ERK2-induced catalytic activation of PAC-1, as measured by *p*-NPP hydrolysis, is not dependent on phosphorylation of ERK2, agreeing with that of MKP-3.[9] Moreover, PAC-1 is capable of dephosphorylating mono-phosphorylated ERK2 (Figure 1(a), and data not shown). Thus, the complete loss of catalytic activation of PAC-1 by alanine or lysine mutation of Arg294 or Arg295 cannot be explained as merely due to a loss of the phosphothreonine binding. Rather, it is possible that ERK2-induced structural rearrangements of

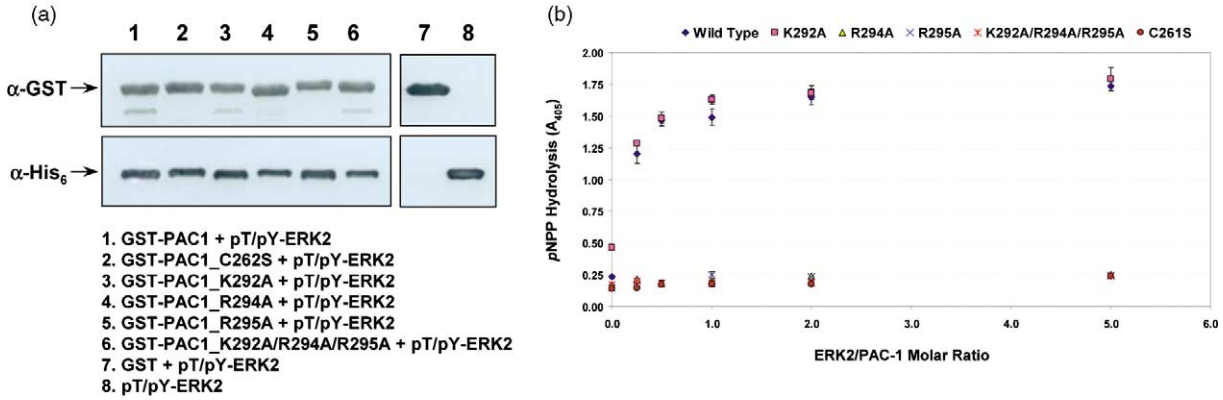

Figure 5. Effects of the active site mutations on ERK2 binding and ERK2-induced catalytic activation of PAC-1. (a) Western blotting analysis showing binding of the wild-type GST-PAC-1 and the active site mutants to the dual-phosphorylated His_6-tagged ERK2. PAC-1/ERK2 binding was visualized by Western blot using anti-His_6-tag antibody (lower upper). Relatively equal amount of GST-PAC-1 proteins or GST used in each experiment was shown by Western blot using anti-GST antibody (upper panel). (b) Effects of PAC-1 mutations on its catalytic activation by ERK2. The phosphatase activity of the wild-type PAC-1 or the active site mutants was measured in *p*-NPP hydrolysis in the presence of the indicated amount of the purified ERK2 for ~2 h at 37 °C, as described in detail in Experimental Procedures.

the phosphatase active site, required for the catalytic activation of PAC-1, are altered as a result of the point mutation of Arg294 or Arg295. This hypothesis is consistent with our NMR structural analysis of the PAC-1 phosphatase domain,[18] which revealed that the α4/α5 loop harboring Arg294 and Arg295 is highly flexible in the free form (Figure 3(a)), suggesting that this region of the enzyme undergoes conformational exchange. Therefore, these results together with those described above argue that, in addition to coordination of the phosphothreonine in the dual-phosphorylated ERK2, the conserved Arg294 and Arg295 likely participate in restructuring of the enzyme active site residues required for the ERK2-induced catalytic activation.

Discussion

Proper regulation of MAPK inactivation is as important as that for its activation in cell growth and differentiation, as well as in cell-growth suppression and apoptosis. Recent studies show that, unlike many other protein phosphatases, catalytic activity of the dual-specificity MAPK phosphatases is controlled by their ability to recognize selective MAPKs and by catalytic activation upon binding to their physiological MAPK substrates.[9] Such a direct coupling of inactivation of an MAPK to activation of an MKP provides a tightly balanced regulatory mechanism that enables these two key enzymes to keep each other in check, thus guaranteeing high fidelity of MAPK-mediated cell signaling. Because of their biological importance in cell signaling, gene expression of MKPs is regulated tightly in cells. For instance, it is known that expression of nuclear-specific MKPs is controlled by immediately early genes induced by activated MAPKs upon nuclear entrance. A recent study has demonstrated that PAC-1 is also transcriptionally induced by the human tumor-suppressor protein p53 in the cell's response to oxidative stress and thus acts as a key downstream effector of p53 function to suppress the MAPK cascade and to induce cell-growth suppression and apoptosis.[34]

Here, we have characterized the MAPK substrate specificity of PAC-1 with recombinant and purified proteins according to three key criteria that are important for structure–function of MKPs: (1) MAPK/MKP binding; (2) MAPK-induced catalytic activation; and (3) dephosphorylation of both the phosphotyrosine and phosphothreonine of an MAPK. Our results suggest strongly that ERK2 is a physiological substrate for PAC-1, because it meets all three criteria. Particularly, ERK2 forms a tight complex with PAC-1 (Figure 2) through its interaction with the amino-terminal domain of PAC-1, as the carboxy-terminal phosphatase domain alone would not be able to form a stable complex with ERK2.[18] The amino-terminal domain of PAC-1 interacts with ERK2 through the conserved KIM sequence, as mutation of the absolutely conserved Arg62 in this sequence abrogated PAC-1/ERK2 association completely (Figure 2(a)). Formation of the PAC-1/ERK2 complex with 1:1 stoichiometry causes catalytic activation of PAC-1, as illustrated by *p*-NPP hydrolysis (Table 1 and Figure 2(f)), which is required for its dual-specificity phosphatase activity for dephosphorylation of ERK2. These characteristics of selective substrate binding and catalytic activation of PAC-1 by ERK2 are reminiscent of those of the cytosolic MKP-3 by ERK2,[9] further supporting that ERK2 is a physiological substrate for PAC-1. Our conclusion is, therefore, consistent with the original discovery of PAC-1 as an MKP in inactivating ERK1/2 in T-cell activation.[30,31]

From our *in vitro* study presented here, it is clear that JNK2 is not a physiological substrate for PAC-1, as it does not interact with or stimulate the catalytic

activity of the phosphatase (Figure 2), nor was JNK2 dephosphorylated after extensive treatment of PAC-1 (Figure 1). Our results agree with a previously reported *in vivo* cell transfection study, which shows the inability of PAC-1 to inactivate the ability of JNK2 to phosphorylate the kinase's substrate c-Jun in NIH3T3 and HeLa cells.[37] On the other hand, our *in vitro* results for PAC-1 with p38α are intriguing. PAC-1 can form a stable complex with p38α (Figure 2(b)), which is accomplished collectively by three arginine residues (Arg62, Arg61 and Arg60) in the KIM sequence (with relative contributions to the affinity of ERK2 binding in the order of Arg62 > Arg61 > Arg60). However, this direct binding does not result in dephosphorylation of p38α by PAC-1 (Figure 2(b)), nor could it cause any catalytic activation of PAC-1, as assessed by the *p*-NPP hydrolysis assay (Table 1). A similar behavior of p38α has been reported in a recent study with another nuclear-specific MKP-2.[10] In that study, it was shown that p38α could form a tight complex with MKP-2 through binding to the amino-terminal domain, but could not stimulate any significant catalytic activation of MKP-2.

In contrast to the inability of PAC-1 to dephosphorylate p38α effectively *in vitro*, it has been shown that transfection of PAC-1 in NIH3T3 and HeLa cells could cause inactivation of p38α activity to phosphorylate its substrate ATF2,[37] suggesting that perhaps PAC-1 could dephosphorylate p38α *in vivo*. One possibility to reconcile our *in vitro* PAC-1 results with the previously reported *in vivo* data is that p38α dephosphorylation and inactivation by PAC-1 might be assisted by another protein that co-associate with them *in vivo*. This hypothesis might be consistent with the notion that catalytic activities of MKP3 and PP2A are regulated by its interaction with protein kinase CK2α.[38,39] Alternatively, the lack of PAC-1 activity towards p38 could be due to the fact that the concentration of the dual-phosphorylated p38 is much lower than its K_m by PAC-1.

Our mutational analysis supports the suggestion that the conserved Arg294 and Arg295 of PAC-1 are important for binding to the phosphothreonine of ERK2.[18] While these conservative mutations did not cause any significant structural perturbations of the enzyme (Figure 3(c)) or reduce the affinity of the PAC-1/ERK2 binding (Figure 5(a)), mutations of these Arg residues to alanine or lysine, either individually or collectively, result in a nearly complete loss of ERK2-induced catalytic activation of PAC-1 (Table 1 and Figure 5(b)) and its ability to dephosphorylate ERK2 (Figure 4(b)–(d)). These results argue that, in addition to phosphothreonine recognition, these two Arg residues may play an important role in substrate-induced structural rearrangement of the phosphatase active site that is required for the optimal phosphatase activity. Indeed, structural analyses of the carboxy-terminal phosphatase domains of PAC-1[18] and MKP-3[17] show that these MKPs, in their free form, exist in a low-activity state because of disengagement of the active site residues. Particularly, the catalytically important general-acid residue Asp230 in PAC-1 or Asp262 in MKP-3 is separated spatially from the other catalytic residues by as much as 10 Å, suggesting that a major conformational change would have to take place in order to bring the catalytic residues together for full enzymatic activity in catalysis. Moreover, our recent NMR binding study shows that the α4/α5 loop of PAC-1 harboring the two Arg residues is involved directly in interactions with the inter-domain sequence of the MKP, which has been implicated to be important for ERK2 binding.[11,13]

In conclusion, our results imply that amino acid residues located at the phospho-amino acid binding sites in the free PAC-1 are structurally disoriented for catalysis, and binding of a specific MAPK restructures the phosphatase active site geometry that is required for PAC-1 optimal catalytic activity for dephosphorylation of the substrate MAPK. Such cooperative pairing of substrate recognition and catalytic activation of an MKP, as seen here in PAC-1, may provide another level in regulation that controls the catalytic activity of an MKP for dephosphorylation and inactivation of a selective MAPK, thereby guaranteeing the fidelity of MAPK functions in cell signaling.

Experimental Procedures

DNA constructs

cDNA encoding the full-length mouse PAC-1 consisting of 318 amino acid residues was amplified by PCR from a mouse spleen cDNA library (Invitrogen), and subcloned into a bacterial expression vector pGEX4T1 (Amersham Bioscience) between BamHI and XhoI sites. All vectors expressing mutant PAC-1 proteins were prepared with a QuikChange site-directed mutagenesis kit (Stratagene). The plasmid co-expressing His_6-tagged ERK2 and a constitutively active MEK1-R4F, which phosphorylates ERK2 upon expression, was received as a generous gift from Melanie Cobb. Mouse p38α cDNA was amplified from HA-p38α in pcDNA3 and subcloned into a modified pET32a vector between BamHI and XhoI sites. The resulting recombinant p38α would contain two extra amino acid residues, Gly-Ser, at the N terminus when the His-tag is cleaved by thrombin. The cDNA encoding human JNK2 was prepared from a HA-JNK2 construct by inserting a PCF fragment between NdeI and BamHI sites of a pET15b expression vector (Novagen). Three extra amino acid residues, Gly-Ser-His, would be added at the N terminus of JNK2 after the N-terminal His-tag in the recombinant protein is removed with thrombin treatment. The HA-p38α plasmid and the HA-JNK2 constructs were kindly provided by Mitchell Goldfarb and Ze'ev Ronai, respectively. Human MKP-5 is cloned between BamHI and EcoRI sites of the bacterial expression pGEX4T1

vector. All cDNA constructs generated in this study were confirmed by sequencing.

Preparation of recombinant proteins

The recombinant full-length wild-type or mutant PAC-1 proteins were expressed in *Escherichia coli* BL21(DE3) cells grown in LB medium at 37 °C for 3–4 h. Protein expression was induced at 18 °C overnight with ~0.4 mM isopropylthio-β-D-galactopyranoside (IPTG). The GST-PAC-1 was purified with glutathione-Sepharose affinity resin (Amersham Biosciences), followed by gel-filtration chromatography. The His_6-tagged MAPK proteins were expressed in *E. coli* BL21(DE3) cells and purified by affinity chromatography on a Hi-Trap nickel column (Pharmacia). The recombinant ERK2 was dual-phosphorylated upon expression by the co-expressed constitutively active MEK1-R4F.[6,40] Phosphorylation of p38α and JNK1/2 was achieved by incubating an MAPK with a GST-fusion MKK4 in a 25 mM Tris–HCl buffer (pH 8.0), containing 0.62 mM EGTA, 10 mM $MgCl_2$, 0.25 mM DTT and 1 mM ATP at room temperature for 20 h. GST-MKK4 was removed by mixing the reaction solution with glutathione-Sepharose beads. The phosphorylation state of the MAPKs was confirmed by Western blot analysis using antibodies specific against dual-phosphorylated ERK2, p38α or JNK1/2.

GST pull-down assay

PAC-1 and MAPK binding was assessed in a GST pull-down assay. Briefly, the GST-fusion wild-type or mutant PAC-1 proteins (5 μM) immobilized on the glutathione-Sepharose beads was incubated with a purified and dual-phosphorylated MAPK (10 μM) in a binding buffer of pH 7.3, containing 140 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, 1.8 mM KH_2PO_4, 2 mM DTT and 2 mM EDTA at 25 °C for 30 min. The glutathione-Sepharose beads (10 μl) were washed extensively with the phosphate-binding buffer. Proteins eluted from the Sepharose beads were separated by SDS-PAGE, and visualized by staining with Coomassie brilliant blue or by Western blotting using anti-GST antibody (Amersham) or anti-histidine tag antibody (Amersham), and alkaline phosphatase-conjugated donkey anti-mouse IgG (Amersham). The dual-phosphorylation state of MAPKs was confirmed by Western blotting using anti-pY, anti-pT MAPK, anti-ACTIVE ERK1/2, anti-ACTIVE p38 and anti-ACTIVE JNK antibodies (Promega).

Phosphatase assays

Dephosphorylation of MAPKs by PAC-1 or MKP-5 was performed by incubating an MAPK (2.5 μM) with PAC-1 or MKP-5 (2.5 μM) in a reaction buffer of 50 mM Tris–HCl (pH 7.5), containing 10 mM $MgCl_2$ and 5 mM DTT at 37 °C for 0–60 min as indicated in the Figure legends. The amounts of each protein and phosphorylation state of MAPKs were assessed by Western blot analysis using specific antibodies as described above. PAC-1 phosphatase activity was measured in a *p*-nitrophenyl phosphate (*p*-NPP) hydrolysis assay. Briefly, 10 μg of a GST-fusion PAC-1 protein was incubated with various amounts of MAPKs with a molar ratio of PAC-1 to MAPK from 1:0 to 1:5 in a 280 μl reaction mixture in a 50 mM Tris–HCl (pH 7.1), containing 100 mM NaCl, 1 mM DTT, 1 mM EDTA and 30 mM *p*-NPP for 2 h at 37 °C. The *p*-NPP hydrolysis reactions were stopped by adding 800 μl of 1 M NaOH, and the reaction product of *p*-nitrophenyl oxide was measured by absorbance at 405 nm. The reverse-phase HPLC analysis of the ERK2 peptide reaction products was conducted with an HP 1050 instrument equipped with a diode array detector. The HPLC analysis was conducted with an ODS-Hypersil column (Keystone Scientific) of 250 mm × 4.6 mm using a gradient of 5%–60% acetonitrile/water in the presence of 0.1% (v/v) trifluoroacetic acid. The flow rate was 1.00 ml/min and the signals were detected with λ at 214 nm. The ERK2 peptides were obtained from PeptidoGenic Research, Inc. (Livermore, CA). Mass spectrometry analysis was performed at the Protein Core Facility at Columbia University.

Preparation of pY-ERK2, pT-ERK2 and ERK2

To prepare pY-ERK2, pT-ERK2 and ERK2, pTpY-ERK2 (0.5 mg in 500 μl) was treated by either serine/threonine phosphatase PP2A (10 μl, 1.3 units) (Upstate) and/or 10 μl of tyrosine-specific HePTP (final concentration 5 μM) (generously provided by Dr Thomas Mustelin) for 1 h at 30 °C. The PP2A and HePTP-treated pTpY-ERK2 yielded pY-ERK2, pT-ERK2 and ERK2, respectively, which were confirmed by Western blot using anti-phosphotyrosine, anti-pT-MAPK and anti-active MAPK antibodies (Promega).

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2005.10.006

The supplementary material consists of a Figure illustrating the protein expression and purification of the full-length mouse PAC-1.

References

1. Lewis, T. S., Shapiro, P. S. & Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Advan. Cancer Res.* **74**, 49–139.
2. Robinson, M. J. & Cobb, M. H. (1997). Mitogen-activated protein kinase pathways. *Curr. Opin. Cell Biol.* **9**, 180–186.
3. Cobb, M. H. & Goldsmith, E. (1995). How MAP kinases are regulated. *J. Biol. Chem.* **270**, 14843–14846.
4. Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell*, **80**, 179–185.
5. Gupta, S., Campbell, D., Derijard, B. & Davis, R. J. (1995). Transdcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, **267**, 389–393.
6. Canagarajah, B. J., Khokhlatchev, A., Cobb, M. H. & Goldsmith, E. J. (1997). Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. *Cell*, **90**, 859–869.
7. Clarke, P. R. (1994). Switching off MAP kinases. *Curr. Biol.* **4**, 647–650.
8. Keyse, S. M. (1994). An emerging family of dual specificity MAPK kinase phosphatases. *Biochim. Biophys. Acta*, **1265**, 152–160.
9. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C. *et al*. (1998). Catalytic activation of the phosphatase MKP-3 by ERK2 mitogen-activated protein kinase. *Science*, **280**, 1262–1265.
10. Chen, P., Hutter, D., Yang, X., Gorospe, M., Davis, R. J. & Liu, Y. (2001). Discordance between the binding affinity of mitogen-activated protein kinase subfamily members for MAP kinase phosphatase-2 and their ability to activate the phosphatase catalytically. *J. Biol. Chem.* **276**, 29440–29449.
11. Farooq, A., Chaturvedi, G., Mujtaba, S., Plotnikova, O., Zeng, L., Dhalluin, C. *et al*. (2001). Solution structure of ERK2 binding domain of MAPK phosphatase MKP-3: Structural insights into MKP-3 activation by ERK2. *Mol. Cell*, **7**, 387–399.
12. Tanoue, T., Adachi, M., Moriguchi, T. & Nishida, E. (2000). A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nature Cell Biol.* **2**, 110–116.
13. Zhou, B., Wu, L., Shen, K., Zhang, J., Lawrence, D. S. & Zhang, Z.-Y. (2001). Multiple regions of MAP kinase phosphatase 3 are involved in its recognition and activation by ERK2. *J. Biol. Chem.* **276**, 6506–6515.
14. Slack, D. N., Seternes, O. M., Gabrielsen, M. & Keyse, S. M. (2001). Distinct binding determinants for ERK2/p38alpha and JNK map kinases mediate catalytic activation and substrate selectivity of map kinase phosphatase-1. *J. Biol. Chem.* **276**, 16491–16500.
15. Fjeld, C. C., Rice, A. E., Kim, Y., Gee, K. R. & Denu, J. M. (2000). Mechanistic basis for catalytic activation of mitogen-activated protein kinase phosphatase 3 by extracellular signal-regulated kinase. *J. Biol. Chem.* **275**, 6749–6757.
16. Zhao, Y. & Zhang, Z. Y. (2001). The mechanism of dephosphorylation of extracellular signal-regulated kinase 2 by mitogen-activated protein kinase phosphatase 3. *J. Biol. Chem.* **276**, 32382–32391.
17. Stewart, A. E., Dowd, S., Keyse, S. K. & McDonald, N. Q. (1999). Crystal structure of the MAPK phosphatase Pyst1 catalytic domain and implications for regulated activation. *Nature Struct. Biol.* **6**, 174–181.
18. Farooq, A., Plotnikova, O., Chaturvedi, G., Yan, S., Zeng, L., Zhang, Q. & Zhou, M.-M. (2003). Solution structure of the MAPK phosphatase PAC1 catalytic domain: insights into substrate-induced enzymatic activation of MKP. *Structure*, **11**, 155–164.
19. Hof, P., Pluskey, S., Dhe-Paganon, S., Eck, M. J. & Shoelson, S. E. (1998). Crystal structure of the tyrosine phosphatase SHP-2. *Cell*, **92**, 441–450.
20. Muda, M., Boschert, U., Dickinson, R., Martinou, J.-C., Martinou, I., Camps, M. *et al*. (1996). MKP-3, a novel cytosolic protein-tyrosine phosphatase that examplifies a new class of mitogen-activated protein kinase phosphatase. *J. Biol. Chem.* **271**, 4319–4326.
21. Groom, L. A., Sneddon, A. A., Aleesi, D. R., Down, S. & Keyse, S. M. (1996). Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. *EMBO J.* **15**, 3621–3632.
22. Muda, M., Boschert, U., Smith, A., Antonsson, B., Gillieron, C., Chabert, C. *et al*. (1997). Molecular cloning and functional characterization of a novel mitogen-activated protein kinase phosphatase, MKP-4. *J. Biol. Chem.* **272**, 5141–5151.
23. Tanoue, T., Moriguchi, T. & Nishida, E. (1999). Molecular cloning and characterization of a novel dual specificity phosphatase MKP5. *J. Biol. Chem.* **274**, 19949–19956.
24. Tanoue, T., Yamamoto, T., Maeda, R. & Nishida, E. (2001). A novel MAPK phosphatase MKP-7 acts preferentially on JNK/SAPK and p38 alpha and beta MAPKs. *J. Biol. Chem.* **276**, 26629–26639.
25. Muda, M., Theodosiou, A., Rodrigues, N., Boschert, U., Camps, M., Gillieron, C. *et al*. (1996). The dual specificity phosphatase M3/6 and MKP-3 are highly selective for inactivation of distinct mitogen-activated protein kinases. *J. Biol. Chem.* **271**, 27205–27208.
26. Keyes, S. M. & Emslie, E. A. (1992). Oxidative stress and heat shock induce a human gene encloding a protein tyrosine phosphatase. *Nature*, **359**, 644–647.
27. Charles, C. H., Abler, A. S. & Lau, L. F. (1992). cDNA sequence of a growth factor-inducible immediate early gene and characterization of its encoded protein. *Oncogene*, **7**, 187–190.
28. Guan, K. L. & Butch, E. (1995). Isolation and characterization of a novel dual specific phosphatase, HVH2, which selectively dephosphorylates the mitogen-activated protein kinase. *J. Biol. Chem.* **270**, 7197–7203.
29. King, A. G., Ozanne, B. W., Smythe, C. & Ashworth, A. (1995). Isolation and characterization of a uniquely regulated threonine, tyrosine phosphatase (TYP1) which inactivates ERK2 and p54jnk. *Oncogene*, **11**, 2553–2563.
30. Rohan, P. J., Davis, P., Moskaluk, C. A., Kearns, M., Krutzsch, H., Siebenlist, U. & Kelly, K. (1993). PAC-1: a mitogen-induced nuclear protein tyrosine phosphatase. *Nature*, **259**, 1763–1766.
31. Ward, Y., Gupta, S., Jensen, P., Wartmann, M., Davis, R. J. & Kelly, K. (1994). Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature*, **367**, 651–654.
32. Kwak, S. P. & Dixon, J. E. (1995). Nultiple dual specificity protein tyrosine phosphatases are expressed and regulated differentially in liver cell lines. *J. Biol. Chem.* **270**, 1156–1160.
33. Ishibashi, T., Bottaro, D. P., Michieli, P., Kelley, C. A. & Aaronson, S. A. (1994). A novel dual specificity

phosphatase induced by serum stimulation and heat shock. *J. Biol. Chem.* **269**, 29897–29902.
34. Yin, Y., Liu, Y. X., Jin, Y. J., Hall, E. J. & Barrett, J. C. (2003). PAC1 phosphatase is a transcription target of p53 in signalling apoptosis and growth suppression. *Nature*, **422**, 527–531.
35. Muda, M., Theodosiou, A., Gilleron, C., Smith, A., Chabert, C., Camps, M. *et al*. (1998). The mitogen-activated protein kinase phosphatase-3 N-terminal noncatalytic region is responsible for tight substrate binding and enzymatic specificity. *J. Biol. Chem.* **273**, 9323–9329.
36. Schumacher, M. A., Todd, J. L., Rice, A. E., Tanner, K. G. & Denu, J. M. (2002). Structural basis for the recognition of a bisphosphorylated MAP kinase peptide by human VHR protein phosphatase. *Biochemistry*, **41**, 3009–3017.
37. Chu, Y., Solski, P. A., Khosravi-Far, R., Der, C. J. & Kelly, K. (1996). The mitogen-activated protein kinase phosphatases PAC1, MKP-1, and MKP-2 have unique substrate specificities and reduced activity *in vivo* toward the ERK2 sevenmaker mutation. *J. Biol. Chem.* **271**, 6497–6501.
38. Castelli, M., Camps, M., Gillieron, C., Leroy, D., Arkinstall, S., Rommel, C. & Nichols, A. (2004). MAP kinase phosphatase 3 (MKP3) interacts with and is phosphorylated by protein kinase CK2alpha. *J. Biol. Chem.* **279**, 44731–44739.
39. Halfar, K., Rommel, C., Stocker, H. & Hafen, E. (2001). Ras controls growth, survival and differentiation in the *Drosophila* eye by different thresholds of MAP kinase activity. *Development*, **128**, 1687–1696.
40. Khokhlatchev, A., Xu, S., English, J., Wu, P., Schaefer, E. & Cobb, M. H. (1997). Reconstitution of MAP kinase phosphorylation cascades in bacteria: efficient synthesis of active protein kinases. *J. Biol. Chem.* **272**, 11057–11062.

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