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Structural basis of p38 α regulation by hematopoietic tyrosine phosphatase

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Abstract

MAP kinases regulate essential cellular events, including cell growth, differentiation and inflammation. The solution structure of a complete MAPK–MAPK-regulatory protein complex, p38 α –HePTP, was determined, enabling a comprehensive investigation of the molecular basis of specificity and fidelity in MAPK regulation. Structure determination was achieved by combining NMR spectroscopy and small-angle X-ray scattering data with a new ensemble calculation–refinement procedure. We identified 25 residues outside of the HePTP kinase interaction motif necessary for p38 α recognition. The complex adopts an extended conformation in solution and rarely samples the conformation necessary for kinase deactivation. Complex formation also does not affect the N-terminal lobe, the activation loop of p38 α or the catalytic domain of HePTP. Together, these results show how the downstream tyrosine phosphatase HePTP regulates p38 α and provide for fundamentally new insights into MAPK regulation and specificity.

Mitogen-activated protein kinases (MAPKs; ERK, p38, JNK) are serine/threonine kinases that mediate the cellular response to a variety of extracellular stimuli ranging from growth factors to inflammatory cytokines. The activity of MAPKs is finely tuned in a cell type–specific and temporal manner by the concerted effort of multiple regulatory proteins, including activating kinases (such as MEK1, MKK3 and MKK6), deactivating phosphatases (such as KIM-PTPs and MKPs) and scaffolding proteins (such as KSRs)¹. Critically, disruptions in the tight regulation of the MAPK signaling pathways are directly correlated with numerous diseases, including Alzheimer’s disease, rheumatoid arthritis and cancer².

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Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>. * Correspondence and requests for materials should be addressed to R.P. or W.P. rebecca_page@brown.edu or wolfgang_peti@brown.edu.

Author contributions D.M.F. and D.K. cloned and purified all proteins. D.M.F. and W.P. performed and analyzed all NMR measurements. D.M.F., W.P. and R.P. performed and analyzed the SAXS measurements. D.K. performed all ITC measurements. R.P. and W.P. helped design the biochemical and NMR experiments. B.R. and G.H. performed all EROS and EROS-NMR calculations. R.P. and W.P. wrote the paper and all authors discussed the results and commented on the manuscript.

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Accession codes. Protein Data Bank: the previously published structures of p38 alone, in the presence of SB203580 and in complex with BIRB796 are deposited under accession codes 1P38, 1A9U and 1KV2, respectively. Biological Magnetic Resonance Bank: chemical shifts recorded in this study are deposited under accession number 17471.

The MAPK pathways consist of three components: a MAP kinase kinase kinase (MAP3K), a MAP kinase kinase (MAP2K) and a MAPK³. Stimulation of the pathway ultimately results in MAPK activation by the dual phosphorylation of a threonine and a tyrosine residue (Thr-Xaa-Tyr) located in the MAPK activation loop. Though the kinase cascades that direct MAPK activation seem stereotypical, the guidance and fine-tuning that direct cell- and situation-specific responses are governed by MAPK-specific phosphatases. In particular, MAPKs are dephosphorylated by the kinase interaction motif protein tyrosine phosphatases (KIM-PTPs), 11 ‘typical’ MAP kinase phosphatases (MKPs) and several ‘atypical’ dual-specific phosphatases as well as serine/threonine phosphatases⁴. The reason for this abundance of phosphatases is most likely related to the many roles that MAPKs play in the cell, which is profoundly affected by the duration of MAPK activation and the subcellular localization of active MAPKs. A comprehensive understanding of how the activity of MAPKs is finely controlled by MAPK regulatory proteins can only be obtained by understanding how these proteins interact at a molecular level.

MAPKs have a two-lobed architecture with a five-stranded β -sheet in the N-terminal lobe and six α helices in the C-terminal lobe⁵. The activation loop, which contains the Thr-Xaa-Tyr motif, is at the top of the C-terminal domain immediately adjacent to the N-terminal domain. In 1994, it was discovered that MAPKs contain a docking site in the C-terminal lobe⁶. This docking site is located on the face of the C-terminal lobe opposite to the activation loop and binds a conserved 13- to 16-amino-acid sequence known as the kinase interaction motif (KIM) or the D motif. This motif has since been identified in nearly every MAPK regulatory and substrate protein. The KIM is characterized by a group of basic residues (two to three lysines or arginines) and two hydrophobic residues (Φ_A -Xaa- Φ_B) that mediate the interaction with the KIM binding site of MAPKs. Structures of MAPKs bound to KIM peptides from activating kinases, deactivating phosphatases and substrates have revealed new insights into the molecular determinants that direct these interactions^{7–13}. However, though the KIM is required for binding^{14,15}, it has recently become clear that residues outside the KIM are necessary to confer specificity^{16–18}.

The KIM-PTPs are a small family of phosphatases that include hematopoietic tyrosine phosphatase (HePTP; immune system-specific)¹⁹, striatum-enriched phosphatase (STEP; brain-specific)²⁰ and STEP-like PTP (PTPRR or PTP-SL; brain-specific)²¹. Each phosphatase possesses a C-terminal catalytic domain (the PTP domain) and an N-terminal unstructured extension that contains the 15-amino-acid KIM. KIM-PTPs regulate the activity of p38 and ERK by both protein localization (by retaining the MAPKs in the cytosol) and dephosphorylation of their active sites²². Despite a wealth of crystallographic data for p38 (refs. 5,7,8,23,24), little is known about the atomic-level detail of its interaction with regulatory proteins outside the KIM binding pocket and the role that these residues have in distinguishing between MAPK regulatory proteins to ensure pathway specificity. Though current structures provide clues to the conformational variability in p38 in the free and KIM peptide-bound forms, additional structures are necessary to obtain comprehensive structural models that describe the molecular interactions between MAPKs and the full-length regulatory proteins that define MAPK pathway specificity and fidelity.

Here, we combine biomolecular NMR spectroscopy, small-angle X-ray scattering (SAXS) and isothermal titration calorimetry (ITC) to determine how p38 α , the p38 isoform that is highly and ubiquitously expressed in most cell types, is recognized by its KIM-PTP regulator HePTP under functional conditions. We show that, although the KIM peptide is essential for binding, p38 α residues outside the KIM binding pocket play a central role in HePTP recognition. We also show that the interaction between p38 α and HePTP is largely confined to the unstructured HePTP N terminus and the KIM-PTP catalytic-domain extension. Finally, using SAXS and a new ensemble-calculation routine that incorporates

chemical-shift information derived from NMR spectroscopy, we show that the complex adopts an extended conformation in solution and provide an atomic model of the p38 α –HePTP complex. Collectively, these results provide new insights into the molecular interactions that regulate the strength and duration of MAPK signaling and, in turn, provide unique avenues for therapeutic interventions of MAPK-related diseases.

RESULTS

Resting-state p38 α analysis

NMR spectroscopy is a powerful technique for defining the interactions of proteins at atomic resolution. However, a precise definition of these interactions requires a highly complete sequence-specific backbone assignment of the protein (or proteins) under investigation. Because of their large size (~40 kDa) and complex dynamic characteristics, serine/threonine kinases are notoriously difficult targets for NMR spectroscopy, and only a few of them, such as the serine/threonine kinase PKA^{25,26} and the tyrosine kinase Abl²⁷, have been investigated in detail using this technique. This is especially true for the MAPKs, for which only two sequence-specific backbone assignments have been reported^{28,29}. However, unlike the assignments for PKA (80% complete)³⁰ or Abl (96% complete)³¹, the assignment of p38 α is only partially complete (64% complete)²⁹. Moreover, many functionally important residues are unassigned.

To further our understanding of these previously unassigned regions of p38 α , we improved the sequence-specific backbone assignment of p38 α to 82% (Fig. 1a–d). Of the 334 expected peaks for nonproline residues, 297 peaks were observed in the two-dimensional ¹H, ¹⁵N transverse relaxation-optimized spectroscopy (TROSY) spectrum of p38 α . The 37 peaks not observed could be missing because of fast solvent (H₂O)-amide exchange on the NMR timescale; lack of D₂O back exchange after protein growth in D₂O-based expression medium, especially for amino acids in the hydrophobic core; or intermediate conformational exchange, which broadens the peaks beyond detection. To minimize solvent-amide exchange, all experiments were performed at pH 6.8 using water suppression by gradient-tailored excitation (WATERGATE)–based NMR experiments. In addition, analysis of unassigned residues suggested that only a small fraction of residues are sufficiently buried in the hydrophobic core to be missing as a result of slow D₂O back exchange. Thus, the majority of missing peaks are probably in a microsecond-millisecond conformational exchange regime that broadens the peaks beyond detection.

Most newly assigned residues are located in functionally critical regions of p38 α (Fig. 1b). The current assignment now includes 88% of the N-terminal lobe, including all of the GxGxxG ATP binding motif; 83% of the activation loop, which contains the Thr-Gly-Tyr phosphorylation site; 100% of the Thr-Gly-Tyr motif; 74% of the MAPK insert (residues 243–273), which includes helices α 1L14 and α 2L14 and forms a C-terminal domain binding pocket that is currently being exploited for the development of new p38-specific inhibitors³²; 100% of the α EF- α F loop, which forms a platform on which the activation loop folds; 100% of helix α H; and 93% of the KIM-peptide binding pocket. Taken together, this is the most complete sequence-specific backbone assignment of any MAPK reported to date.

The assignment of residues surrounding the ATP binding pocket were confirmed by titration of both 2',3'-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid ester) adenosine triphosphate (SL-ATP)^{33,34}, a spin-labeled ATP analog, and the prototypical p38 α inhibitor SB203580 (ref. 35), a competitive inhibitor of ATP that binds at the ATP binding site, into [²H, ¹⁵N]p38 α (two- to four-fold excess of titrant) using two-dimensional ¹H, ¹⁵N-TROSY spectroscopy. As expected, only residues surrounding the ATP binding pocket showed either reduced intensities (SL-ATP; Supplementary Results, Supplementary Fig. 1) or large

chemical shift changes (SB203580; Fig. 1c). In addition, titration with SB203580 did not change the number of observed peaks (296 peaks), demonstrating that the microsecond-millisecond intermediate timescale dynamics of p38 α are unchanged when it is bound to SB203580.

Notably, the largest contiguous patch of unassigned p38 α residues connects the N- and the C-terminal lobes of p38 α (Supplementary Fig. 2a,b). This connection is mediated by the DFG loop and stretches into the MAPK insert in the C-terminal lobe. Titration with BIRB796 (Fig. 1d), a prototypical DFG-out inhibitor that binds and stabilizes the DFG loop in the inactive 'out' conformation³⁶, resulted not only in chemical shift perturbations of residues near the BIRB796 binding site but also in altered microsecond-millisecond intermediate timescale p38 α dynamics, as a total of 15 additional peaks were observed in the spectrum. Thus, BIRB796 changes the conformational equilibrium of all DFG loop residues, including Phe169 (ref. 36), as well as additional residues in this region (Supplementary Fig. 2c). This is notable as this finding correlates well with a recently introduced model for the catalytic reactions of kinases that is based on hydrophobic spines that coordinate internal kinase dynamics³⁷. The hydrophobic spine, the R-spine (Phe169, unassigned; Leu75, unassigned; Leu86, assigned; His148, assigned), is centered on the DFG loop. Our data show that stabilization of Phe169 (for example, by an inhibitor such as BIRB796) and, in turn, other spine residues such as Leu86 influence kinase dynamics.

The p38 α docking site extends past the KIM binding pocket

We used NMR spectroscopy to define the complete p38 α -HePTP resting-state complex interface at atomic resolution (Fig. 2a-e). In this experiment, [²H,¹⁵N]p38 α was incubated with unlabeled HePTP (constructs used in this study are illustrated in Fig. 2a), the 78-kDa p38 α -HePTP complex was purified by size-exclusion chromatography and a two-dimensional ¹H,¹⁵N-TROSY spectrum of the complex was recorded. The complex interacts tightly ($K_d = 2.5 \pm 0.6 \mu\text{M}$; Table 1 and Supplementary Fig. 3) and is stable for days at 23 °C. The spectrum is of excellent quality for a complex of this size (78 kDa), allowing the chemical shift perturbations in p38 α caused by HePTP binding to be readily detected (Fig. 2b).

Direct comparison of the two-dimensional ¹H,¹⁵N-TROSY spectra of free and HePTP-bound p38 α revealed chemical shift perturbations in 45 peaks, 34 of which were more than 4 s.d. (σ) above the mean (Fig. 3a,b). As expected, a subset of the chemical shift perturbations were localized to the KIM binding pocket, including helix α D, the α D- α E loop, the β 7- β 8 reverse turn, the ED site and the CD site (the latter two sites are often collectively referred to as the acidic binding pocket). However, perturbation of residues that are distal to the KIM binding pocket were also detected, including multiple residues from the α C- β 4 loop, the α E- β 7 loop, helix α F and the α F- α G loop. To our knowledge, this demonstrates for the first time that residues outside the p38 α KIM binding pocket contribute to HePTP interaction and thus are important for binding.

KIM interactions with MAPKs are determined by the KIM

The structure of the HePTP_{KIM} peptide (HePTP residues 16–31) in complex with ERK2 (ref. 13), as well as three structures of KIM peptides (one with MEF2a and two with MKK3b) bound to p38 α (ref. 7,8), have been reported. In the p38 α -KIM peptide complexes, no interaction of the peptides with the CD domain (Asp313 and Asp316) was observed. In contrast, the HePTP_{KIM} peptide makes multiple contacts with the CD domain of ERK2. Our chemical shift perturbation data with the p38 α -HePTP complex unequivocally confirm the interaction of HePTP with the p38 α CD and ED domains in solution, which involves CD domain residues Gln310–Asp313, Asp315–Glu317 (Pro314 is

undetectable in the two-dimensional ^1H , ^{15}N -TROSY spectrum) and ED residues Glu160 and Asp161. Thus, HePTP clearly interacts with both the ED and the CD sites in the p38 α -HePTP complex and, based on the observed chemical shift changes, most likely adopts a conformation similar to that observed in the ERK2-HePTP_{KIM} peptide complex. This demonstrates a critical point for MAPK selectivity: the sequence of the KIM, and not the identity of the MAPK, defines how KIM peptides from different MAPK effector proteins bind and interact with MAPKs.

To identify the residues of HePTP interacting with p38 α , we isolated the individual domains of HePTP that most likely contribute to p38 α binding, namely the KIM peptide (HePTP_{KIM31}; Fig. 2a) and the HePTP catalytic domain (HePTP_{CAT}; Fig. 2a). We then used NMR chemical shift perturbation mapping experiments to determine which of these domains participate in binding. First, we probed the interaction of the HePTP_{KIM31} peptide with p38 α at four different protein-peptide ratios (Supplementary Fig. 4). Upon addition of the HePTP_{KIM31} peptide to [^2H , ^{15}N]p38 α , 35 large chemical shift changes corresponding to residues in the p38 α C lobe and Leu108 in the hinge region were observed. Consistent with the p38 α -HePTP complex chemical shift perturbation experiment, no substantial chemical shift differences were observed for peaks corresponding to residues in the p38 α N-lobe (Figs. 2c and 3a,b). Remarkably, the total number of perturbed peaks was lower in the titration of p38 α with HePTP_{KIM31} than in the p38 α -HePTP complex. Whereas complex formation between p38 α and HePTP resulted in large chemical shift changes in multiple residues from helix αD , the αD - αE loop, the $\beta 7$ - $\beta 8$ reverse turn, the ED site and the CD site, titration with HePTP_{KIM31} resulted in fewer shifts in these regions (Fig. 3a,b). Moreover, titration with HePTP resulted in multiple shifts outside the KIM that were not observed with HePTP_{KIM31} alone. This shows that, though HePTP_{KIM31} binds and interacts with p38 α , it does not recapitulate the shifts observed with HePTP, demonstrating that surfaces outside the KIM binding pocket are essential for the formation of the p38 α -HePTP complex.

We also investigated the role of the HePTP residue Val31. Sequence alignments with other MAPK KIMs predicted that the HePTP Φ_{A} -X- Φ_{B} residues correspond to Leu27-Xaa-Leu29. However, the crystal structure of the ERK2-HePTP_{KIM}peptide complex¹³ revealed a register shift in the Φ_{A} -Xaa- Φ_{B} sequence in which Leu27 binds in a third hydrophobic pocket, $\Phi_{\text{A}-2}$, and Leu29 actually binds in the Φ_{A} pocket. Similar $\Phi_{\text{A}-2}$ and Φ_{A} interactions have been seen for the MEF2a peptide with p38 α (ref. 8). Thus, Val31 putatively binds in the Φ_{B} pocket. However, the absence of electron density observed for Val31 indicates flexibility and thus potential dispensability for binding. This is consistent with the observation that ERK2-bound KIM peptide conformations are heterogeneous, with only weak overlap between corresponding Φ_{A} and Φ_{B} residues. In contrast, the KIM peptides bound to p38 α adopt highly similar conformations, and the corresponding Φ_{A} and Φ_{B} residues overlap nearly perfectly (Fig. 4a,b and Supplementary Fig. 5).

Because the Φ_{B} interactions between KIM peptides and p38 α are so highly conserved, we hypothesized that Val31 is not dispensable but instead has an important role in binding to p38 α , which we examined again using NMR chemical shift mapping with the peptide HePTP_{KIM30}, which lacks Val31, at four different protein-peptide ratios (Supplementary Fig. 4). The addition of Val31 resulted in a considerable increase in the number of residues that experienced chemical shift perturbations: from 23 (HePTP_{KIM30}) to 35 (HePTP_{KIM31}) (Fig. 3a,b). The localization of the majority of additional shifts to the Φ_{B} binding pocket indicates that this is the binding site for Val31 (Fig. 4b). Remarkably, the addition of Val31 also resulted in a chemical shift perturbation of Leu108 in the hinge region, which connects the N and C lobes of p38 α . This suggests that the domain closure mediated by the hinge in MAPKs upon KIM-peptide binding most likely requires occupation of the Φ_{B} binding pocket. Unexpectedly, increases in the number of chemical shift perturbations for residues

that contribute to the Φ_{A-2} and Φ_A binding pockets, as well as the acidic binding pocket, were also observed for HePTP_{KIM31}. Taken together, these data suggest that HePTP_{KIM31} interacts more tightly with p38 α than HePTP_{KIM30}. This was confirmed using ITC, which showed that the HePTP_{KIM31} binds p38 α with a K_d of $5.2 \pm 1.3 \mu\text{M}$ (Table 1 and Supplementary Fig. 3), whereas the K_d for the HePTP_{KIM30}-p38 α interaction was sufficiently weak that it could not be accurately determined. These results demonstrate that HePTP_{KIM31} occupies all three hydrophobic binding pockets (Φ_{A-2} , Φ_A and Φ_B) in p38 α and provide additional evidence that the mechanism of MAPK binding is determined by the KIM sequence and not the MAPK.

The p38 α activation loop is unaffected by HePTP binding

Crystallographic studies of MAPK–KIM peptide complexes have also shown that docking interactions alter the structure of the activation loop, resulting in a more ‘active-like’ conformation of the MAPK^{7–13,18}. To determine whether the same changes occur in solution, we examined the chemical shifts of the p38 α activation loop residues (172–183) in p38 α alone and bound to either the HePTP_{KIM31} peptide or the HePTP protein. Remarkably, no chemical shift perturbations were observed for any of the peaks corresponding to activation-loop residues (10 of the 12 residues are assigned, including the Thr-Gly-Tyr motif). This demonstrates that, in solution, binding of HePTP_{KIM31} or HePTP to p38 α does not change the local environment of activation-loop residues.

p38 α does not interact with HePTP_{CAT}

It is well known that the HePTP_{KIM} is necessary for the formation of a stable p38 α –HePTP complex¹⁵. However, it is unknown whether weak NMR-detectable interactions (mM) occur between p38 α and HePTP_{CAT}. Thus, we probed the interaction of p38 α with HePTP_{CAT}, which lacks residues 1–43 but includes HePTP $\alpha 0$, a secondary structural element that is unique to the KIM-PTP family among the classical PTPs³⁸. Surprisingly, we detected absolutely no interactions between p38 α and HePTP_{CAT} (Figs. 2d and 3b). This was unexpected because activated p38 α is dephosphorylated by HePTP¹⁵, and thus, at some point during catalysis, the phosphorylated tyrosine of the p38 α activation loop must bind the HePTP active site. Thus, all chemical shift perturbations observed for the p38 α –HePTP complex are mediated by residues N-terminal to HePTP_{CAT}, namely, the HePTP_{KIM31}, and the residues immediately C-terminal to the HePTP_{KIM31} known as the kinase specificity sequence (KIS), namely, the HePTP_{KIS} (ref. 16).

To confirm this hypothesis, we performed reverse NMR titration experiments with [²H,¹⁵N]HePTP and unlabeled p38 α . The two-dimensional ¹H,¹⁵N-TROSY spectrum of HePTP overlaps nearly perfectly with that of HePTP_{CAT}, demonstrating that there is no interaction between the residues N-terminal to the catalytic domain (residues 15–43) and HePTP_{CAT} (Supplementary Fig. 6a). This allowed us to transfer the previously published assignment of HePTP_{CAT} (ref. 39) to that of HePTP. Additionally, the new peaks in the spectrum, owing to the additional 29 residues at the N terminus, were clustered at ~8.0 p.p.m., a chemical shift range typical for unstructured residues. We also detected chemical shift perturbations for residues 44–56 corresponding to helix $\alpha 0$, which indicate that the flexibility of residues 15–43 alters the chemical environment of residues 44–56.

To test the effect of p38 α binding, the complex between [²H,¹⁵N]HePTP and unlabeled p38 α was formed, and a two-dimensional ¹H,¹⁵N-TROSY spectrum of the 78-kDa complex was collected (Supplementary Fig. 6b). All crosspeaks that clustered at ~8.0 p.p.m. in the free HePTP spectrum were perturbed in the spectrum of the p38 α –HePTP complex, demonstrating that the flexible residues in HePTP, 15–56, bind strongly to p38 α and adopt an ordered conformation. Thus, HePTP residues that mediate strong and specific p38 α

binding include not only the HePTP_{KIM31} peptide but also the HePTP_{KIS}, which includes helix $\alpha 0$ (residues 31–56) and thus HePTP residues 15–56 (Supplementary Fig. 6c).

The HePTP_{KIMKIS} mediates binding to p38 α

To validate these results, we generated the HePTP_{KIMKIS} peptide, which corresponds to HePTP residues 15–56. The HePTP_{KIMKIS} peptide was directly titrated into [²H,¹⁵N]p38 α (it also copurifies with p38 α using size-exclusion chromatography). As predicted by our earlier studies, the chemical shift perturbation mapping of the HePTP_{KIMKIS} peptide bound to p38 α almost completely recapitulated that observed in the p38 α –HePTP complex, demonstrating that all of the interactions between p38 α and HePTP are mediated by the HePTP_{KIMKIS} peptide (Figs. 2e and 3a,b). We also performed a titration with ¹⁵N-labeled HePTP_{KIMKIS} peptide and unlabeled p38 α as an additional experiment to show that all residues of the HePTP_{KIMKIS} peptide bind to p38 α (Supplementary Fig. 7). Notably, the HePTP_{KIMKIS} peptide interaction with p38 α showed a few additional chemical shift perturbations. Correspondingly, it had an approximately three-fold increase in binding affinity over HePTP, as measured by ITC (Table 1 and Supplementary Fig. 3). The additional interaction is most likely due to nonpolar contacts between HePTP_{KIS} hydrophobic residues (Ile40, Val43, Pro46 and Val49) and p38 α residues (Leu217, Thr218, His228, Asn269, Asn272, Phe274, Ile275 and Ala277). These p38 α residues showed larger chemical shift perturbations when bound to the HePTP_{KIMKIS} peptide than when in complex with HePTP. Thus, both NMR and ITC data show that the presence of HePTP_{CAT} slightly weakens the interactions observed for p38 α residues that preferentially interact with the HePTP_{KIMKIS} peptide.

The p38 α –HePTP complex solution structure

SAXS provides information on the global size and shape of a protein in solution. The experimental scattering curve represents the average scattering of all conformations weighted by the number of molecules in each conformational state. We therefore used SAXS to determine the size and shape of the individual p38 α and HePTP proteins and the p38 α –HePTP complex in solution. SAXS profiles ($I(q)$ versus q , with intensity I and scattering vector q) and pair-distribution functions are shown in Figure 5a,b (p38 α –HePTP complex) and Supplementary Figure 8 (p38 α and HePTP alone). The I_0 -to-concentration ratio was consistent for measurements of at least two concentrations, demonstrating no detectable aggregation and thus confirming the high quality of the samples. The experimental radius of gyration (R_g) value of 33.2 ± 0.5 Å for the p38 α –HePTP complex was determined from Guinier analysis ($n = 4$; Supplementary Table 1). As expected, this was considerably larger than that for either protein alone ($R_g = 23.7 \pm 0.4$ Å for p38 α and 24.2 ± 0.2 Å for HePTP). *Ab initio* determination of the molecular envelope also converged on an R_g of 34.1 Å for the complex. These values for R_g are, respectively, 7.2 Å and 8.1 Å larger than what is predicted for a globular protein with the same number of amino acids⁴⁰. Collectively, these results demonstrate that in the resting state the p38 α –HePTP complex is highly extended, with p38 α and HePTP associating in a head-to-head, head-to-tail or tail-to-tail manner.

To further our understanding of the p38 α –HePTP complex, we developed a new computational procedure, ensemble refinement of SAXS (EROS)-NMR, which allowed us to determine the relative orientation of p38 α and HePTP that provides the best fit to the experimental SAXS data. EROS-NMR includes the key features of EROS⁴¹: a coarse-grained model for protein binding is used to simulate the molecular assembly, which generates an initial ensemble of starting protein conformations. This ensemble is subsequently refined to improve the fit to the SAXS data. In addition, EROS-NMR accounts for NMR chemical shift data by incorporating a bias in the initial p38 α –HePTP simulation.

Namely, a nonspecific attractive potential was applied between the HePTP_{KIS} linker (HePTP residues 32–56) and the p38 α residues (Asn114, Asn115, Lys118, Lys121, Ser154, Gly219, Arg220) that fulfill three conditions: they show large chemical shift changes, they are surface exposed and they are not in the vicinity of the p38 KIM binding pocket. The bias potential was weak so that the HePTP_{KIS} linker could bind and unbind repeatedly from p38 α during the course of the simulation. The conformational space occupied by these rigid bodies was thoroughly sampled by simulation to identify conformations that best fit the SAXS data.

The best fit to the experimental data ($\chi^2 = 1.3$) was obtained for an ensemble of four conformations, which includes the single-best-fit model (SBFM) with the highest relative weight (47%), as well as three additional models (relative weights 23%, 16%, 14%; Fig. 5c,d and Supplementary Fig. 9). This fit is considerably better than that observed for the SBFM alone ($\chi^2 = 1.5$), for an ensemble that includes only two models ($\chi^2 = 1.4$), or for ensembles with five or more models, as the inclusion of more models only marginally improves χ^2 . All models in the four-model ensemble are globally similar, with p38 α and HePTP associating in an end-to-end manner such that the HePTP_{CAT} is localized to the bottom of the p38 α C-terminal domain. They only differ in the position of the HePTP_{CAT} domain. In fact, overlaying the models using p38 α suggests that in solution the HePTP_{CAT} fluctuates in a fan-like motion below p38 α about the central SBFM conformation, with the weights of the additional models steadily decreasing as the distance from the SBFM increases (Supplementary Fig. 9). These changes in the position of HePTP_{CAT} are accommodated by changes in the flexible HePTP_{KIS} peptide, which adopts one of two classes of conformations: it either extends back toward the ATP binding pocket before bending down toward the bottom of the p38 α C-terminal domain (the dominant SBFM conformation belongs to this class), or it bends down immediately after the last HePTP_{KIM} residue. Remarkably, in none of the four structures was the active site of HePTP (Cys270) localized near the p38 α activation loop, an interaction that must occur when HePTP dephosphorylates activated p38 α . To quantify this important point, we also performed a full maximum-entropy refinement of 1,000 simulation structures⁴¹. The region of conformational space spanned by the resulting ensemble is consistent with that spanned by the smaller four-model ensemble, with, at most, 1% of structures in which the C α atoms Tyr182 (p38 α) and Cys270 (HePTP) are within 18 Å of each other (Supplementary Fig. 10). Notably, the corresponding structures extend the fan-like motion previously seen for the four-conformer ensemble toward the p38 α activation loop (Supplementary Fig. 9). Thus, under resting-state conditions, HePTP and p38 α adopt an elongated end-to-end complex that rarely (<1%) samples the orientation required for dephosphorylation.

DISCUSSION

It is commonly accepted that KIM peptides are only partially responsible for recognition of effector proteins by MAPKs, as multiple studies have shown that additional interactions between MAPK effectors and substrates are required to maintain the exquisite specificity observed in MAPK signaling pathways^{16–18}. Here, we have combined the molecular detail provided by NMR spectroscopy with the overall molecular organization afforded by SAXS to determine which residues outside the HePTP_{KIM31} direct and stabilize the resting-state conformation of the p38 α –HePTP complex. One of the central findings of this study is that the HePTP_{KIM31} peptide alone is not able to recapitulate the interactions observed in the p38 α –HePTP complex. Instead, multiple NMR titration experiments of p38 α with HePTP constructs of varying lengths and domain architectures, coupled with ITC, reveal that both the HePTP_{KIM31} and HePTP_{KIS} sequences are necessary and sufficient to mediate all binding interactions with p38 α . To our knowledge, this is the first time that residues outside a KIM of a MAPK effector protein have been determined at the molecular level.

Using SAXS, we have also shown that in solution the interactions mediated by the HePTP_{KIMKIS} lead to the formation of a p38 α –HePTP complex in which p38 α and HePTP interact in an elongated, end-to-end manner. This was unexpected because phosphorylated p38 α is dephosphorylated by HePTP, which requires the formation of a more globular complex. However, it is clear from our SAXS studies that under resting-state conditions the p38 α –HePTP complex is not globular but instead is highly elongated. This result differs considerably from that of a recently published crosslinking study⁴² in which a resting-state PTP–SL–ERK2 complex was crosslinked and the structure of the complex was modeled using the crosslinking distances as constraints. Jointly, these results demonstrate that the overall orientation of MAPK–KIM–PTP resting-state complexes may differ depending on the MAPK, the KIM–PTP or both.

To generate a structural model for the p38 α –HePTP complex in solution, we developed a new SAXS refinement procedure, EROS-NMR, which incorporates NMR chemical shift data into a recently developed SAXS refinement procedure. Incorporation of the NMR chemical shift data ensures that the models are consistent with not only the SAXS data, which define overall shape, but also the NMR chemical shift data, which define molecular interactions that direct the organization of the complex. As expected on the basis of multiple failed attempts to crystallize the resting-state complex, the SAXS refinements show that the complex is dynamic, as four models provide a better fit to the SAXS data than a single model. The models in the four-model ensemble have similar overall conformations, which differ primarily in the relative orientation of the HePTP_{CAT} below the p38 α C-terminal domain. The HePTP_{KIS} conformation is variable, but adopts highly similar conformations as it projects downward from the p38 α KIM binding pocket. The largest differences are observed in the C-terminal portion of the HePTP_{KIS}, which adopts an extended conformation and interacts differentially with p38 α in the four ensemble models of the p38 α –HePTP complex. This shows that the N-terminal HePTP_{KIS} residues interact directly with p38 α , whereas the C-terminal HePTP_{KIS} residues transiently bind p38 α , allowing additional conformations to be sampled about the central dominant conformation (the single-best-fit conformation). This model is also consistent with a slight decrease in binding affinity between HePTP_{KIMKIS} and HePTP with p38 α , as measured by ITC, showing that this flexibility slightly lowers the overall strength of binding.

Taken together, these results provide fundamental new insights into the molecular basis of MAPK regulation and specificity. As additional similar studies are completed, the subset of residues outside the KIM binding pocket that are specific for different MAPK–MAPK regulatory protein complexes will be determined and, in turn, will allow for the development of new drug therapies that target these specific complexes.

METHODS

Sample preparation

The HePTP_{KIM31} peptide (VRLQERRGSNVALMLDV) was synthesized and HPLC purified (> 98% purity; Biosynthesis); the HePTP_{KIM30} peptide (RLQERRGSNVALMLD) was synthesized and HPLC purified (>98% purity; gift from J. Offer, University of Cambridge). Expression and purification of HePTP and HePTP_{CAT} were carried out as previously described^{14,38} except that the final size-exclusion chromatography (SEC) step was carried out using p38 α protein buffer (50 mM HEPES, pH 6.8, 150 mM NaCl, 5 mM dithiothreitol). p38 α and the HePTP_{KIMKIS}^{C42S} peptide were subcloned into a derivative of the pET28 vector that includes an N-terminal His₆ tag and a TEV protease–recognition sequence⁴³. Unlabeled proteins were expressed in BL21-(DE3) RIL *Escherichia coli* cells (Stratagene) in LB medium. [²H,¹⁵N]p38 α and HePTP were expressed in M9 medium supplemented with ¹⁵NH₄Cl (1 g l^{−1}) in 99% D₂O, and [²H,¹³¹⁵N]p38 α was expressed in

M9 medium supplemented with $^{15}\text{NH}_4\text{Cl}$ (1 g l^{-1}) and $^{13}\text{C}_6\text{-d}_7\text{-glucose}$ (4 g l^{-1}) in 99% D_2O . p38 α and the HePTP_{KIMKIS} were purified using standard protocols (Supplementary Methods). For p38 α –HePTP complex formation, p38 α was incubated with HePTP (1:1 molar ratio) on ice for 45 min and purified using SEC equilibrated in p38 α protein buffer. The p38 α –HePTP complex elutes at the position expected for the p38 α –HePTP heterodimer. The ^{2}H , ^{15}N]p38 α –HePTP complex and the p38 α – ^{2}H , ^{15}N]HePTP complex were concentrated to 0.2 mM and stored on ice (<8 h) until NMR data acquisition.

p38 α sequence-specific backbone assignment

All NMR experiments were performed at 298 K on a Bruker Advance II 800 MHz spectrometer equipped with a TCI HCN z -gradient cryoprobe. NMR samples were prepared in protein buffer in 90%/10% (v/v) $\text{H}_2\text{O}/\text{D}_2\text{O}$. The NMR spectra were processed with Topspin 2.1 (Bruker) and analyzed using the CARA software package (<http://www.nmr.ch/doku.php>). Purified ^{2}H , ^{13}C , ^{15}N]p38 α was concentrated to 0.58 mM and stored on ice (<8 h) until data acquisition. The sequence-specific backbone assignment was obtained from TROSY versions of the following experiments: two-dimensional ^1H , ^{15}N -TROSY, three-dimensional HNCA, three-dimensional HNCACB, three-dimensional HN(CO)CA, three-dimensional HN(CO)CACB and a three-dimensional ^{15}N -edited ^1H , ^1H -NOESY spectrum. The assignment of residues around the ATP binding pocket were confirmed by titration of 2',3'-(2,2,5,5-tetramethyl-3-pyrroline-1-oxyl-3-carboxylic acid ester) adenosine triphosphate (SL-ATP, a generous gift from P. Vogel, Southern Methodist University)³⁴ into ^{2}H , ^{13}C , ^{15}N]p38 α ; final ratios were 0.16 mM p38 α /0.32 mM SL-ATP (1:2) and 0.16 mM p38 α /0.64 mM SL-ATP (1:4). Two-dimensional ^1H , ^{15}N -TROSY spectra were recorded. All quantitative spin-label experiments were processed with NMRPipe⁴⁴, and peak integrals were determined using CARA (<http://www.nmr.ch/doku.php>).

p38 α –HePTP NMR interaction studies

Both HePTP_{KIM31} and HePTP_{KIM30} peptides were solubilized in protein buffer. Experiments were performed at ratios of 1:1, 1:3, 1:5 and 1:8 excess peptide, which were added to ^{2}H , ^{15}N]p38 α , and a two-dimensional ^1H , ^{15}N -TROSY spectrum was recorded (100 μM p38 α ; 1:1 experiment was also recorded at 250 μM p38 α). A two-fold excess of unlabeled HePTP_{CAT} was added to ^{2}H , ^{15}N]p38 α (0.2 mM p38 α /0.4 mM HePTP_{CAT}), and a two-dimensional ^1H , ^{15}N -TROSY spectrum was recorded. An equal molar ratio of unlabeled HePTP_{KIMKIS} was added to ^{2}H , ^{15}N]p38 α (94 μM p38 α /94 μM HePTP_{KIMKIS}), and a two-dimensional ^1H , ^{15}N -TROSY spectrum was recorded. The ^{2}H , ^{15}N] p38 α –unlabeled HePTP complex was purified and concentrated to 0.2 mM, and a two-dimensional ^1H , ^{15}N -TROSY spectrum was recorded. p38 α –HePTP_{KIM30} titrations in ratios of 1:1, 1:3, 1:5 and 1:8 were used to follow chemical shift changes in p38 α upon binding, as nearly all peaks showed fast exchange and thus could be easily followed. This data was used for the analysis of the 1:1-, 1:3-, 1:5- and 1:8-ratio p38 α –HePTP_{KIM31} titrations and all other interactions (HePTP_{KIMKIS} and HePTP), allowing for high confidence of peak assignments of p38 α in the bound state. All chemical shift differences ($\Delta\delta$) were calculated using the following equation:

$$\Delta\delta \text{ (p.p.m.)} = \sqrt{(\Delta\delta_{\text{H}})^2 + \left(\frac{\Delta\delta_{\text{N}}}{10}\right)^2}$$

SAXS structural refinement

We adapted the EROS method for the refinement of SAXS data⁴¹ to incorporate NMR data. EROS was previously used to determine the structure of protein kinase C in solution⁴⁵, and

a related method has been used to refine SAXS data of Hck kinase⁴⁶. In the simulations of the p38 α -HePTP complex, a nonspecific, attractive potential was applied between the KIS linker (HePTP residues 32–56) and the p38 α residues (Asn114, Asn115, Lys118, Lys121, Ser154, Gly219, Arg220) that fulfill three conditions: they show large chemical shift changes, they are surface exposed and they are not in the vicinity of the p38 KIM binding pocket. The bias potential increased the well depth of the amino acid interactions by only 1 $k_B T$, such that the KIS linker could easily bind and unbind from p38 α during the course of the simulation. The scattering intensities $I_k(q)$ were calculated for all simulated structures k , and the ensemble intensity was defined as

$$I^{\text{sim}}(q) = \sum_{k=1}^n w_k I_k(q)$$

where w_k is the relative weight assigned to structure k in an ensemble of n structures. The difference between computed and measured intensities was quantified by

$$\chi^2(\{w_k\}) = N^{-1} \sum_{i=1}^N \left[a I^{\text{sim}}(q_i) + b - I^{\text{exp}}(q_i) \right]^2 / \sigma^2(q_i)$$

where N is the number of data points and a and b are fitting parameters obtained from conditions $\chi^2/a = \chi^2/b = 0$. The parameter a sets the intensity scale and the parameter $b \ll I^{\text{exp}}(q=0)$ is a small background correction. In the SAXS profile calculations, the hydration-shell electron-density contrast ρ was also varied, and best fits were obtained for $\rho = 0.005 \text{ e}/\text{\AA}^3$. To fit the experimental data with a small number of simulation structures, we defined a function $G = \chi^2 + \mu n$, which was minimized numerically by varying the number of structures n with nonzero weights and their weights w_k under the constraint $\sum_k w_k = 1$. Here $\mu > 0$ is a control parameter that captures our preference for having a small number n of structures in the ensemble, analogous to a chemical potential. As μ is decreased, n increases and the value of χ^2 decreases upon minimization of G . Beyond $n = 4$, the quality of the fit does not improve substantially (with $\chi^2 = 1.3, 1.4$ and 1.5 for $n = 4, 2$ and 1 , respectively). For reference, we also performed a full maximum-entropy refinement over the entire ensemble⁴¹, which produced an ensemble consistent with the region of conformation space spanned by the set of $n = 4$ structures.

Other methods

Methodological details of additional p38 α -HePTP interaction studies, isothermal titration calorimetry and SAXS are described in Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Raman M, Chen W, Cobb MH. Differential regulation and properties of MAPKs. *Oncogene*. 2007; 26:3100–3112. [PubMed: 17496909]
2. Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta*. 2010; 1802:396–405. [PubMed: 20079433]
3. Cobb MH, et al. Regulation of the MAP kinase cascade. *Cell. Mol. Biol. Res.* 1994; 40:253–256. [PubMed: 7874203]
4. Alonso A, et al. Protein tyrosine phosphatases in the human genome. *Cell*. 2004; 117:699–711. [PubMed: 15186772]
5. Wilson KP, et al. Crystal structure of p38 mitogen-activated protein kinase. *J. Biol. Chem.* 1996; 271:27696–27700. [PubMed: 8910361]
6. Kallunki T, et al. JNK2 contains a specificity-determining region responsible for efficient c-Jun binding and phosphorylation. *Genes Dev.* 1994; 8:2996–3007. [PubMed: 8001819]
7. Akella R, Min X, Wu Q, Gardner KH, Goldsmith EJ. The third conformation of p38 α MAP kinase observed in phosphorylated p38 α and in solution. *Structure*. 2010; 18:1571–1578. [PubMed: 21134636]
8. Chang CI, Xu BE, Akella R, Cobb MH, Goldsmith EJ. Crystal structures of MAP kinase p38 complexed to the docking sites on its nuclear substrate MEF2A and activator MKK3b. *Mol. Cell*. 2002; 9:1241–1249. [PubMed: 12086621]
9. Heo YS, et al. Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125. *EMBO J.* 2004; 23:2185–2195. [PubMed: 15141161]
10. Liu S, Sun JP, Zhou B, Zhang ZY. Structural basis of docking interactions between ERK2 and MAP kinase phosphatase 3. *Proc. Natl. Acad. Sci. USA*. 2006; 103:5326–5331. [PubMed: 16567630]
11. Ma W, et al. Phosphorylation of DCC by ERK2 is facilitated by direct docking of the receptor P1 domain to the kinase. *Structure*. 2010; 18:1502–1511. [PubMed: 21070949]
12. Reményi A, Good MC, Bhattacharyya RP, Lim WA. The role of docking interactions in mediating signaling input, output, and discrimination in the yeast MAPK network. *Mol. Cell*. 2005; 20:951–962. [PubMed: 16364919]
13. Zhou T, Sun L, Humphreys J, Goldsmith EJ. Docking interactions induce exposure of activation loop in the MAP kinase ERK2. *Structure*. 2006; 14:1011–1019. [PubMed: 16765894]
14. Critton DA, Tortajada A, Stetson G, Peti W, Page R. Structural basis of substrate recognition by hematopoietic tyrosine phosphatase. *Biochemistry*. 2008; 47:13336–13345. [PubMed: 19053285]
15. Saxena M, Williams S, Brockdorff J, Gilman J, Mustelin T. Inhibition of T cell signaling by mitogen-activated protein kinase-targeted hematopoietic tyrosine phosphatase (HePTP). *J. Biol. Chem.* 1999; 274:11693–11700. [PubMed: 10206983]
16. Muñoz JJ, Tarrega C, Blanco-Aparicio C, Pulido R. Differential interaction of the tyrosine phosphatases PTP-SL, STEP and HePTP with the mitogen-activated protein kinases ERK1/2 and p38 α is determined by a kinase specificity sequence and influenced by reducing agents. *Biochem. J.* 2003; 372:193–201. [PubMed: 12583813]
17. Tanoue T, Adachi M, Moriguchi T, Nishida E. A conserved docking motif in MAP kinases common to substrates, activators and regulators. *Nat. Cell Biol.* 2000; 2:110–116. [PubMed: 10655591]
18. Zhang J, Zhou B, Zheng CF, Zhang ZY. A bipartite mechanism for ERK2 recognition by its cognate regulators and substrates. *J. Biol. Chem.* 2003; 278:29901–29912. [PubMed: 12754209]
19. Zanke B, et al. Cloning and expression of an inducible lymphoid-specific, protein tyrosine phosphatase (HePTPase). *Eur. J. Immunol.* 1992; 22:235–239. [PubMed: 1530918]
20. Lombroso PJ, Murdoch G, Lerner M. Molecular characterization of a protein-tyrosine-phosphatase enriched in striatum. *Proc. Natl. Acad. Sci. USA*. 1991; 88:7242–7246. [PubMed: 1714595]

21. Hendriks W, Schepens J, Brugman C, Zeeuwen P, Wieringa B. A novel receptor-type protein tyrosine phosphatase with a single catalytic domain is specifically expressed in mouse brain. *Biochem. J.* 1995; 305:499–504. [PubMed: 7832766]
22. Saxena M, Williams S, Tasken K, Mustelin T. Crosstalk between cAMP-dependent kinase and MAP kinase through a protein tyrosine phosphatase. *Nat. Cell Biol.* 1999; 1:305–311. [PubMed: 10559944]
23. Bellon S, Fitzgibbon MJ, Fox T, Hsiao HM, Wilson KP. The structure of phosphorylated p38 γ is monomeric and reveals a conserved activation-loop conformation. *Structure.* 1999; 7:1057–1065. [PubMed: 10508788]
24. Diskin R, Engelberg D, Livnah O. A novel lipid binding site formed by the MAP kinase insert in p38 α . *J. Mol. Biol.* 2008; 375:70–79. [PubMed: 17999933]
25. Masterson LR, et al. Dynamics connect substrate recognition to catalysis in protein kinase A. *Nat. Chem. Biol.* 2010; 6:821–828. [PubMed: 20890288]
26. Masterson LR, Mascioni A, Traaseth NJ, Taylor SS, Veglia G. Allosteric cooperativity in protein kinase A. *Proc. Natl. Acad. Sci. USA.* 2008; 105:506–511. [PubMed: 18178622]
27. Zhang J, et al. Targeting Bcr-Abl by combining allosteric with ATP-binding-site inhibitors. *Nature.* 2010; 463:501–506. [PubMed: 20072125]
28. Piserchio A, et al. Solution NMR insights into docking interactions involving inactive ERK2. *Biochemistry.* 2011; 50:3660–3672. [PubMed: 21449613]
29. Vogtherr M, et al. NMR backbone assignment of the mitogen-activated protein (MAP) kinase p38. *J. Biomol. NMR.* 2005; 32:175. [PubMed: 16034669]
30. Esposito V, et al. NMR assignment of the cAMP-binding domain A of the PKA regulatory subunit. *J. Biomol. NMR.* 2006; 36(suppl. 1):64. [PubMed: 17016670]
31. Vajpai N, et al. Backbone NMR resonance assignment of the Abelson kinase domain in complex with imatinib. *Biomol. NMR Assign.* 2008; 2:41–42. [PubMed: 19636920]
32. Perry JJ, Harris RM, Moiani D, Olson AJ, Tainer JA. p38 α MAP kinase C-terminal domain binding pocket characterized by crystallographic and computational analyses. *J. Mol. Biol.* 2009; 391:1–11. [PubMed: 19501598]
33. Hoffman AD, Urbatsch IL, Vogel PD. Nucleotide binding to the human multidrug resistance protein 3, MRP3. *Protein J.* 2010; 29:373–379. [PubMed: 20563633]
34. Delannoy S, Urbatsch IL, Tomblin G, Senior AE, Vogel PD. Nucleotide binding to the multidrug resistance P-glycoprotein as studied by ESR spectroscopy. *Biochemistry.* 2005; 44:14010–14019. [PubMed: 16229490]
35. Fitzgerald CE, et al. Structural basis for p38 α MAP kinase quinazolinone and pyridol-pyrimidine inhibitor specificity. *Nat. Struct. Biol.* 2003; 10:764–769. [PubMed: 12897767]
36. Vogtherr M, et al. NMR characterization of kinase p38 dynamics in free and ligand-bound forms. *Angew. Chem. Int. Edn Engl.* 2006; 45:993–997.
37. Kornev AP, Haste NM, Taylor SS, Eyck LF. Surface comparison of active and inactive protein kinases identifies a conserved activation mechanism. *Proc. Natl. Acad. Sci. USA.* 2006; 103:17783–17788. [PubMed: 17095602]
38. Mustelin T, Tautz L, Page R. Structure of the hematopoietic tyrosine phosphatase (HePTP) catalytic domain: structure of a KIM phosphatase with phosphate bound at the active site. *J. Mol. Biol.* 2005; 354:150–163. [PubMed: 16226275]
39. Jeeves M, McClelland DM, Barr AJ, Overduin M. Sequence-specific ¹H, ¹³C and ¹⁵N backbone resonance assignments of the 34 kDa catalytic domain of human PTPN7. *Biomol. NMR Assign.* 2008; 2:101–103. [PubMed: 19636879]
40. Gong H, Freed KF. Electrostatic solvation energy for two oppositely charged ions in a solvated protein system: salt bridges can stabilize proteins. *Biophys. J.* 2010; 98:470–477. [PubMed: 20141761]
41. Ró ycki B, Kim YC, Hummer G. SAXS ensemble refinement of ESCRT-III CHMP3 conformational transitions. *Structure.* 2011; 19:109–116. [PubMed: 21220121]
42. Balasu MC, et al. Interface analysis of the complex between ERK2 and PTP-SL. *PLoS ONE.* 2009; 4:e5432. [PubMed: 19424502]

43. Peti W, Page R. Strategies to maximize heterologous protein expression in *Escherichia coli* with minimal cost. *Protein Expr. Purif.* 2007; 51:1–10. [PubMed: 16904906]
44. Delaglio F, et al. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J. Biomol. NMR.* 1995; 6:277–293. [PubMed: 8520220]
45. Leonard TA, Rozycki B, Saidi LF, Hummer G, Hurley JH. Crystal structure and allosteric activation of protein kinase C β II. *Cell.* 2011; 144:55–66. [PubMed: 21215369]
46. Yang S, Blachowicz L, Makowski L, Roux B. Multidomain assembled states of Hck tyrosine kinase in solution. *Proc. Natl. Acad. Sci. USA.* 2010; 107:15757–15762. [PubMed: 20798061]
47. Franke D, Svergun DI. DAMMIF, a program for rapid *ab-initio* shape determination in small-angle scattering. *J. Appl. Crystallogr.* 2009; 42:342–346.

Figure 1. The sequence-specific backbone assignment of p38 α is the most complete of any MAPK (a) Fully annotated two-dimensional ^1H , ^{15}N -TROSY spectrum of p38 α recorded at 298K on a Bruker AvanceII 800 MHz spectrometer. Assigned peaks are labeled with the corresponding residue (single-letter code) and number in the protein sequence. (b) Surface representation of assigned residues. Unassigned residues are shaded light gray, and assigned residues are shaded dark gray, blue (cd site), orange (KIM binding pocket), green (activation loop, including the Thr-Gly-Tyr motif) and yellow (MAPK insert) (PDB code: 1P38). (c) Titration of the p38 α ATP competitive inhibitor SB203580 results in chemical shift perturbations localized to the residues that comprise the ATP binding site. Left, histogram showing the combined $^1\text{H}/^{15}\text{N}$ chemical shift perturbations versus residue. Residues corresponding to the glycine-rich loop and N- and C-terminal hinge are indicated by dashed lines. Right, surface representation of the p38 α residues that show chemical shift perturbations in the presence of SB203580 (PDB code: 1A9U; SB203580 shown in orange sticks). Residues with chemical shift perturbations $>2\sigma$ are colored light blue, those $>3\sigma$ are colored slate blue and those $>4\sigma$ are colored raspberry. (d) Titration of the p38 α DFG-out inhibitor BIRB796 results in chemical shift perturbations localized to the residues near the BIRB796 binding site between the N- and C-terminal domains; histogram and surface representation of observed perturbations are identical to those described in c (PDB code: 1Kv2; BIRB796 shown as orange sticks).

Figure 2. 2D [¹H, ¹⁵N]-TROSY spectra obtained for [²H, ¹⁵N]p38α in complex with various HePTP domains

(a) Constructs used in the present study. (b) Two-dimensional ¹H, ¹⁵N-TROSY spectrum of p38α (black, 40 kDa) overlaid with that of the p38α–HePTP complex (red; 78 kDa; 1:1 ratio; complex purified by SEC immediately before measurements). (c) p38α titrated with HePTP_{KIM31} (1:8 ratio; red). (d) p38α titrated with HePTP_{CAT} (1:2 ratio, red). (e) p38α titrated with HePTP_{KIMKIS} (1:1 ratio, red).

Figure 3. The p38 α binding site for HePTP extends beyond the KIM binding pocket
 Residue-specific perturbations of p38 α with the HePTP_{KIM30} peptide (1:8 ratio), the HePTP_{KIM31} peptide (1:8 ratio), the HePTP_{KIMKIS} peptide (1:1 ratio), HePTP_{CAT} (1:2 ratio) or HePTP (1:1 ratio; complex purified by SEC immediately before measurements). **(a)** Residues that show chemical shift perturbations greater than 2σ are illustrated in a surface representation and colored according to the legend. **(b)** Histograms showing the combined $^1\text{H}/^{15}\text{N}$ chemical shift perturbations versus residue number. The cutoffs for deviations larger than 2σ (light blue), 3σ (slate blue) and 4σ (raspberry) are indicated by vertical lines. Residues corresponding to the CD and ED sites are indicated by dashed lines. Peaks corresponding to MAPK insert residues Leu262–Gln264 disappeared independent of titrant.

Figure 4. Val31 binds hydrophobic pocket Φ_B in p38 α

(a) KIM binding pockets of p38 α (top) and ERK2 (bottom) with p38 α KIM peptides MEF2A (coral), MKK3B (light blue) and MEK3B (slate blue) and with ERK2 peptides DCC (yellow), MKP3 (magenta, light blue) and HePTP (green). The positions of the conserved Φ_A and Φ_B binding pockets are shown. Some KIM peptides, like HePTP (ERK2) and MEF2A (p38 α), also bind a third hydrophobic pocket, Φ_{A-2} . p38 α –KIM and ERK2–KIM complexes superimposed using the C-terminal domains (detailed in Supplementary Fig. 5). (b) Residue-specific perturbations of p38 α with the HePTP_{KIM30} peptide (1:8 ratio; top) or the HePTP_{KIM31} peptide (1:8 ratio; bottom) illustrated using a surface representation of p38 α . The number and magnitude of shifts, especially about the Φ_B binding pocket, are substantially greater when Val31 is present in the peptide. Val31 was not observed in the electron density map of the HePTP–ERK2 complex. However, our chemical shift titration experiments show that Val31 most likely occupies the Φ_B pocket when bound to p38 α (color scheme is the same as in Fig. 3b).

Figure 5. The p38–HePTP resting state complex is extended in solution

(a) Experimental SAXS data ($I(q)$ versus q) of the p38 α –HePTP complex shown as black squares with error bars as gray lines. Error bars show the experimental error based on circular averaging of the two-dimensional solution scattering data. The theoretical scattering curve from the four-model ensemble is shown in red. Inset, Guinier plots for 2 mg ml⁻¹ and 4 mg ml⁻¹. (b) The $P(r)$ function of the SAXS data, where P is the pair-distance distribution function and r is the distance vector. (c) Superposition of the single-best-fit solution to the SAXS data with an *ab initio* molecular envelope generated by DAMMIF⁴⁷, in two views rotated by 90 degrees. (d) The four models that constitute the four-model ensemble, with the percentage that each model contributes to the scattering. The model with the highest contribution to the scattering curve (47%) is also the single-best-fit model. Blue, p38 α ; green, HePTP_{KIM}; magenta, HePTP_{KIS}; orange, HePTP_{CAT}.

Table 1Thermodynamic and dissociation constants for p38 α -HePTP and p38 α -HePTP peptide complexes

Complex	K_d (nM)	ΔH (kcal mol ⁻¹)	$-T\Delta S$ (kcal mol ⁻¹)	ΔG (kcal mol ⁻¹)
p38 α -HePTP	2,570 $\pm$ 650	-20.5 $\pm$ 2.3	12.9 $\pm$ 2.2	-7.6 $\pm$ 0.1
p38 α -HePTP _{KIMKIS}	778 $\pm$ 28	-24.9 $\pm$ 1.0	16.6 $\pm$ 1.0	-8.3 $\pm$ 0.0
p38 α -HePTP _{KIM31}	5,150 $\pm$ 1,290	-19.6 $\pm$ 5.5	12.4 $\pm$ 5.6	-7.2 $\pm$ 0.1

Data are derived from ITC experiments at 25 °C and represent mean values $\pm$ one s.d. for triplicate measurements except for HePTP_{KIM31}, which was performed in duplicate.