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Synthetic Phosphorylation of p38α Recapitulates Protein Kinase Activity

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Supporting Information

ABSTRACT: Through a "tag-and-modify" protein chemical modification strategy, we site-selectively phosphorylated the activation loop of protein kinase p38α. Phosphorylation at natural (180) and unnatural (172) sites created two pure phospho-forms. p38α bearing only a single phosphocysteine (pCys) as a mimic of pThr at 180 was sufficient to switch the kinase to an active state, capable of processing natural protein substrate ATF2; 172 site phosphorylation did not. In this way, we chemically recapitulated triggering of a relevant segment of the MAPK-signaling pathway *in vitro*. This allowed detailed kinetic analysis of global and stoichiometric phosphorylation events catalyzed by p38α and revealed that site 180 is a sufficient activator alone and engenders dominant monophosphorylation activity. Moreover, a survey of kinase inhibition using inhibitors with different (Type I/II) modes (including therapeutically relevant) revealed unambiguously that Type II inhibitors inhibit phosphorylated p38α and allowed discovery of a predictive kinetic analysis based on cooperativity to distinguish Type I vs II.

Protein kinases transfer phosphoryl onto side chains of protein residues (typically Ser, Thr, and Tyr).[1] Their activity is often triggered or modulated by phosphorylation on a so-called activation loop (Figure 1),[2] which causes a change in conformation.[3] In this way, kinases in a specific sequence generate consequential phosphorylation events that lead to amplifying cascades in one of the dominant modes of intracellular signaling and regulation (Figure 1a).[4] p38α mitogen-activated protein kinase (MAPK) is implicated in critical widespread events such as inflammation,[5] somatic,[6] cardiac,[7] and neural[8] cellular regulation. It is the most studied protein kinase[9] and the most targeted therapeutically.[10] Once the MAPK cascade is triggered by external receptor engagement (Figure 1a), "upstream" kinases are activated that in turn activate p38α. This occurs naturally by phosphorylation at Thr180 and Tyr182 in the activation loop (Figure 1b).[11] p38α, in turn, phosphorylates other protein substrates, including activating transcription factor 2 (ATF2), which is implicated in many roles, including pathways that, when disregulated, lead to melanoma.[12]

Such cascades are finely tuned, so small changes can be amplified, leading to ready disregulation and hence pathogenicity. Given that representative biological samples[13] (Supporting Information (SI) Figure S1) are typically heterogeneous[14] (as for many post-translationally modified proteins), alternative strategies for their precise study are required.[15] For detailed *in vitro* studies, such as activity/signaling triggering, a direct non-enzymatic method would be useful. Here we use such a strategy in the chemical recapitulation of an activation−protein phosphorylation sequence from a relevant section of MAPK-mediated signaling (Figure 1a, box).

p38α is difficult to activate non-enzymatically.[16] A common technique for kinases mutates phosphorylation sites to Asp/Glu. However, at physiological pH, the sp^2 carboxylate side chains are poor phospho-mimetics, having not only different pK_a values ($pK_a \approx 4$ C(O)OH, cf. $pK_a1 \sim 2$, $pK_a2 \sim 6$ OP(O)$(OH)_2$)[17] but also insufficient charge (−1 cf. −2) or structural (trigonal planar cf. tetrahedral) mimicry. Indeed, in p38α, successful constitutive activation has only been achieved indirectly by mutating off-target residues to favor the stabilization of an active conformation.[16,18] To our knowledge, recapitulation of effective charge mimicry in p38α has not been possible until now, and this has precluded key hypotheses regarding the effect of point charge localization in such critical signaling cascades.

In vitro, site-selective chemical modification gives an alternative potential strategy. Although an elegant approach for chemical attachment of a single negative charge[19] has shown some modulation of kinase activity in MEK1, the groups used were not phospho-derived or charge-matched and were redox-sensitive, bringing disadvantages associated with non-representative instability, charge, and pK_a. We previously demonstrated the first examples of site-selective, chemical protein phosphorylation,[20] although not in naturally phosphorylated enzymes. This employs a "tag-and-modify" approach, which involves introducing an orthogonally reactive functional group—the tag—that can be used as a selective chemical handle for further modification and introduction of the desired group.[21] Incorporating dehydroalanine (Dha)[20a,22,23] as a tag allows diverse modification,[20,23,24] including phosphorylation.

To test the effect of site-selective chemical phosphorylation of the activation loop in p38α, two sites were selected: 172 and 180—only 180 is naturally phosphorylated (as pThr180). First, a Dha "tag" was site-selectively installed in two different p38α variants (SI sections 4 and 5) at 172 and 180 by treating p38α-

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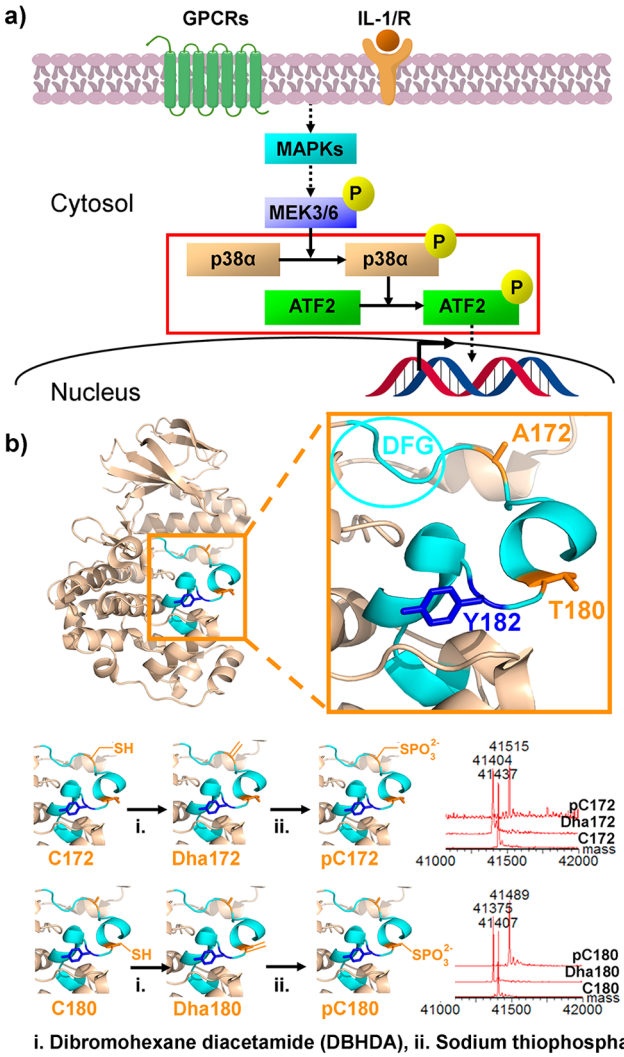
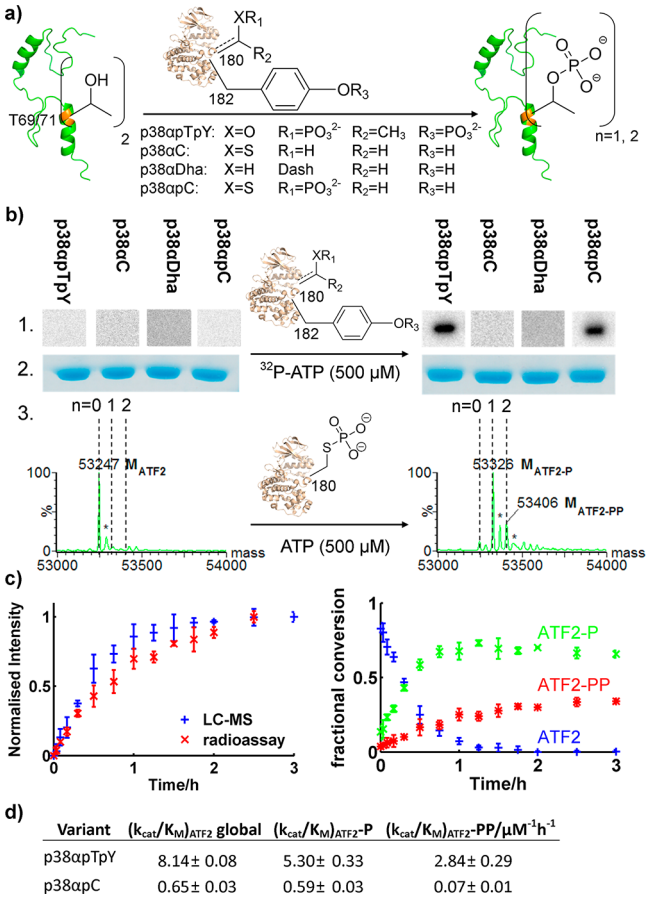


Figure 1. (a) p38α and the MAP kinase cascade (shown here for ATF2 activation). (b) Structure, design, and synthesis of synthetically phosphorylated p38α; box shows sites of natural (180, 182) and unnatural (172) phosphorylation in the activation loop.



d)

Variant	$(k_{cat}/K_M)_{ATF2}$ global	$(k_{cat}/K_M)_{ATF2}$-P	$(k_{cat}/K_M)_{ATF2}$-PP/$\mu M^{-1}h^{-1}$
p38αpTpY	8.14± 0.08	5.30± 0.33	2.84± 0.29
p38αpC	0.65± 0.03	0.59± 0.03	0.07± 0.01

Figure 2. (a) Enzymatic phosphorylation by variants of p38α of ATF2 generated by both chemical and biological methods. (b) 1, Electrophoretic radioassay (ERA); 2, Coomassie stain; and 3, intact protein ESI-MS were used to assay phosphorylation of ATF2. (c) Left: Comparison of a representative time course of global phosphorylation using p38α-pCys180 as enzyme, measured by ERA and MS. Right: MS-allowed assay of precise ATF2 phospho state (representative time course). (d) Quantitative kinetic parameters for global, mono- (P), and di- (PP) phosphorylation of ATF2 derived from these assays.

Cys172 and p38α-Cys180, respectively, with selective reagent 2,5-dibromohexane diacetamide (DBHDA, SI section 3; Figure 1b, step i).[23] Next, reaction of Dha with sodium thiophosphate (Figure 1b, step ii) proceeded cleanly and completely (SI section 5) to create phospho-amino acid phosphocysteine (pCys), to yield p38α-pCys172 and p38α-pCys180, respectively. Protein characterization confirmed generation of the intended folded p38α pure phospho-forms: peptide mapping using proteolytic digestion and LC-MS/MS (SI section 7 for Cys→Dha→pCys mapping) confirmed chemistry at only intended sites, despite the presence of other native Cys (Cys39, Cys211) in the designed constructs (p38α-C119S:C162S:A172C and -C119S:C162S:T180C), highlighting selectivity based on predicted accessibility (Figure S3). Circular dichroism (CD) spectroscopy revealed that these synthetically phosphorylated p38αs retained correct α-helical/β-sheet structure (SI section 7 and Figure S2) and that their temperature profiles were essentially identical to native.

With pure, synthetic phospho-forms in hand, we characterized catalytic activity (Figure 2). While small peptide substrates allow qualitative assay, useful for high-throughput, our intention in creating homogeneous constructs was to develop precise mechanistic understanding, aided by prior analyses with mixed systems.[13] Use of relevant protein substrate ATF2[25] therefore allowed us to recapitulate a segment of MAPK signaling (Figure 1a) and to consider both global and individual phosphorylation events. This was assessed by both mass spectrometry (MS, Figure 2b,c and SI section 8) and electrophoretic radioassay (ERA, Figure 2b and SI section 9): ERA using [^{32}P]-labeled ATP provides a measure of total phosphorylation, while direct intact protein ESI-MS kinetics allows mono-/di-phosphorylation states of ATF2 to be determined (Figure 2b). Consistent with an anticipated need for activation loop phosphorylation, non-phosphorylated synthetic p38 precursors (p38α-Cys172, p38α-Dha172, p38α-Cys180, p38α-Dha180) showed no phosphorylating activity. Synthetic phosphorylation at natural (180) and unnatural (172) phosphorylation sites, both within the loop, to create p38α-pCys180 and p38α-pCys172, respectively, had dramatically contrasting effects. Although correctly formed, unnaturally phosphorylated p38α-pCys172 was inactive. However, excitingly, phosphorylation at the single natural site 180 in p38α-pCys180 was sufficient to generate clear activity (Figure 2c).

Kinetic characterization of this active, pure, synthetic phospho-form p38α-pCys180 provided key mechanistic insights.

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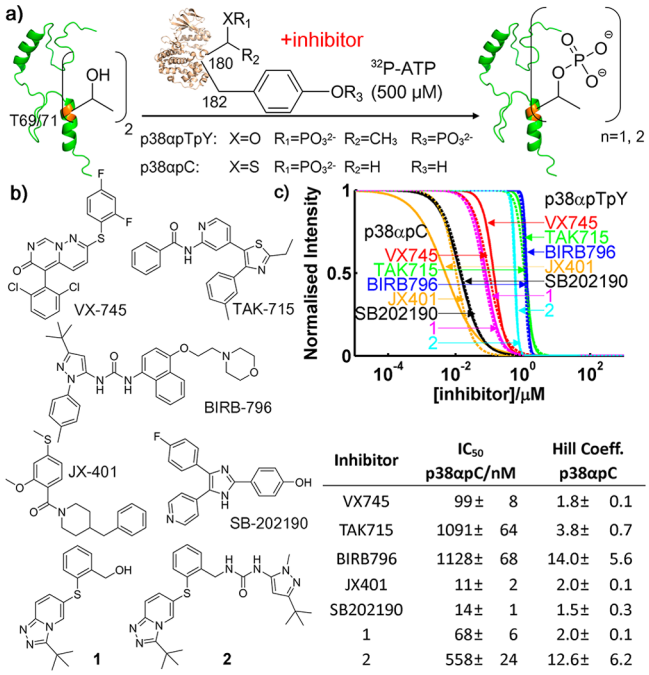
Quantitative analysis revealed that pCys is a sufficient mimic of pThr; it triggers activity (k_{cat}/K_M(global) = 0.65 ± 0.03 μM^{-1} h^{-1}) at a level equal to that created through site Thr180 phosphorylation in biologically derived, mixed samples.[13d] This is ~10% of the activity (Figure 2d and SI sections 8 and 9) of doubly phosphorylated p38α-pThr180-pTyr182 (generated enzymatically[14]), consistent with results from other mixed, biologically derived samples.[13d] This mimicry is successful despite $\beta O \rightarrow \beta S$, likely α-epimers, and lack of Thr Cβ-Me substituent. Lack of activity for p38α-pCys172 shows that correct charge and mimicry in the activation loop are not enough—site is also important. We note this generation of 172-phosphorylated variants (applicable, in principle, to any site) would not be possible with current biological methods.

The MS method used here is a rare example of on-protein modification kinetic determination.[26] Comparison (Figure 2c) revealed good correlation between global ATF2 phosphorylation determined by MS and, more typical, ERA. However, MS also revealed key subtleties in phospho-state: ATF2 is phosphorylated at Thr69 and Thr71. Compared to initial mono- (ATF2-P) and di-phosphorylation (ATF2-PP) rates with p38α-pThr180-pTyr182, those for pure phospho-form p38α-pCys180 show a bias toward ATF2-P. This switch (ATF2-P:ATF2-PP = 1.9→8.9) suggests that phosphate at site 180 engenders critical activation of mono-P kinase activity and that at site 182 then enables di-P. MS^n analysis suggested that ATF2-P is phosphorylated only at Thr69 (SI section 8.6). Moreover, this difference in selectivity highlights that current analyses of protein phosphorylation at a global level may ignore key elements of selectivity, resulting in mechanistic implications in kinase signaling. The design of synthetic kinase (in activated phosphorylated form) used here also maintains an essentially intact, unaltered activation loop. While alterations can be achieved through disruptive mutational analyses[13c,16,27] (e.g., Tyr→Phe182) to "mutate out" phosphorylation sites, it is known that loop residues act in concert,[3] and losses of key functional groups (e.g., Tyr-OH) may create an unclear mechanistic picture.

To test the scope of mechanistic analysis possible with the synthetic system described here, we also evaluated the effect of protein kinase inhibitors as powerful probes of function.[9] These have been characterized on the basis of observations made in inhibitory co-crystal structures, according to induction of so-called DFG-loop (Figure 1) "in" (Type I) or "out" (Type II) states.[28] A representative range of Type I (VX745,[29] TAK715[30] SB202190, and **1**[31]), Type II (BIRB-796[10b] and **2**[31]), and unknown (JX401) inhibitors was tested. The pure phospho-form p38α-Cys180 generated high-quality inhibitory profiles (Figure 3c, SI section 10), consistent with known potencies for Type I. Our data also directly addressed whether Type II inhibitors bind active phosphorylated protein kinase states.[28] It has been suggested that the Type II mode of action may depend on sequestering inactive kinase in DFG-out non-phosphorylated form.[28] However, to be consistent with the Curtin–Hammett principle, this would depend critically on suitable equilibration kinetics. With impure, "phosphorylated" p38α-pThr180-pTyr182 generated by enzymatic activation[14] (>10% non-phospho, only ~30% di-phospho), such a possibility cannot be discounted. Now, through formation of pure active form, we can demonstrate direct inhibition (Figure 3). Moreover, this inhibition was characterized by a marker that distinguished inhibitor types. Under the conditions employed,[28] Type I inhibitors are favored, as reflected in more potent inhibition by Type I inhibitors (Figure 3d) over Type II. However, Type II



Inhibitor	IC_{50} p38αpC/nM		Hill Coeff. p38αpC	
VX745	99±	8	1.8±	0.1
TAK715	1091±	64	3.8±	0.7
BIRB796	1128±	68	14.0±	5.6
JX401	11±	2	2.0±	0.1
SB202190	14±	1	1.5±	0.3
1	68±	6	2.0±	0.1
2	558±	24	12.6±	6.2

Figure 3. (a) Inhibition of p38α-mediated phosphorylation of ATF2. (b) Type I and II inhibitors used. (c) Inhibition–dose curves (dotted for chemically generated p38α-Cys180 and solid for biologically generated[14] p38α-pThr180-pTyr182; see SI section 10 for individual curves). (d) Inhibition data.

showed a clear cooperativity (shown by Hill coefficients) for their inhibition. Large coefficients are typically ignored[32] but may be consistent[33] with conformation-alteration mechanisms (e.g., "DFG-out" of phosphorylated loop triggered by site-directed, localized unfolding from the Type II urea motif). This readily applied measurement might complement current structural distinction of inhibitor type during development.[28] Interestingly, our method also highlights an ambiguity of TAK-715, which displays intermediate characteristics; this has also recently been noted structurally.[34] We also predict JX401 to be a Type I inhibitor.

Finally, to explore the generality of synthetic chemical phosphorylation in other key kinases, we also tested MEK1. Site-selective reaction at activation site 222 successfully gave phospho-form MEK1-pCys222 (SI sections 11 and 12).

In summary, we have rationally designed and constructed synthetic variants of p38α that allow precise recapitulation of activation through chemical phosphorylation. Choice of a natural phosphorylation site seems critical in this kinase; modification causes activation, as would be the case with enzymatic phosphorylation at the same position. Through site-selective phosphorylation at a non-native site, we show that location is key to activity, not simply the addition of charge alone. The methodology for kinase activation described here opens further possibilities for *in vitro* functional studies of other kinases for which it has been difficult to obtain intrinsically active mutants.[24b] Interestingly, differences in tolerance were also noted during our synthetic protein phosphorylation chemistry: p38α-pCys172 was formed from p38α-Dha172 more rapidly than p38α-pCys180 from p38α-Dha180 (SI section 5.5). This is consistent with potential electrostatic interactions[35] (SI Figure S3) and suggests such chemistry might also be used to probe the inherent tolerance of phospho-sites within proteins. The mechanistic origins of the intriguing p38α mono- vs di-

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phosphorylation selectivity we observed here are unknown but may also relate to electrostatic modulation in concert with Tyr182.

Given the mixtures produced by biological methods,[14] this represents a rare, pure phospho-form of a kinase. Other powerful methods, such as protein semi-synthesis or assembly of phosphorylated peptide fragments, could also be considered.[36] Such precise methods should allow further insight into enzyme reaction mechanisms, excluding other confounding factors (e.g., dominant catalyst impurities). For example, the chemically controlled switch-like nature of our method employs substrates (e.g., p38α-Dha180) that can *only* be chemically phosphorylated and so unambiguously remove other possible mechanisms (auto-phosphorylation) of kinase activation.[37] We now have chemical control of the site 180 "on switch" for p38α not found through traditional biology. We also note that, despite extensive structural work, some mechanistically informative kinase structures, such as Type II inhibitors bound to active form, still do not exist, and our strategy for generating pure forms may also facilitate their formation. Cys phosphorylation has also recently been noted in bacterial signaling.[38] In this way, we aim to unlock further details of the key chemical mechanisms behind signaling.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): L.H.J. is an employee and shareholder of Pfizer.

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