Supplementary Information

Selective MAP kinase activity sensors through the application of directionally-programmable D domain motifs

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Peptide synthesis of sensors with reversed docking domains.



The reversed docking motifs, for inclusion in the p38 and ERK sensors, were synthesized via standard SPPS on the mild-acid labile resin, Fmoc-Gly-NovaSyn-TGT (Millipore). To the docking motif, PEG_{2} -propionic acid (AAPPTec) and 6-azido-hexanoic acid were then coupled via standard conditions. The phosphorylation motifs were prepared via SPPS on PAL-PEG-PS (Invitrogen). Following synthesis of the phosphorylation motif through the PEG₂ moiety, propiolic acid was coupled: The dried resin was swelled in anhydrous CH_2Cl_2 and subjected to a mixture of propiolic acid (3 eq.) and EEDQ (3.1 eq.) in anhydrous CH_2Cl_2 (1.0 mL per 100 mg resin). The reaction was allowed to proceed for 3 h. at 25 °C. The resin was then washed 5 X CH_2Cl_2 , 5 X DMF, 5 X CH_2Cl_2 and 5 X MeOH. The resin was then dried under vacuum.

Following the synthesis of the docking motif, the docking motif peptide was cleaved fully protected in 30% hexafluoroisopropanol (HFIP) in CH_2Cl_2 , 4 x 10 min. The resin was then washed with CH_2Cl_2 (4 x 10 min) and washes were combined with cleaved peptide. Solvent was removed in vacuo and the peptide was dried under vacuum.

The crude docking motif (30 μ mol) was then dissolved in anhydrous THF and mixed with Cul (3 eq.), DIPEA (20 eq.), and THF (0.1 M). The peptide mixture was then introduced to the on-resin phosphorylation motif peptide (20 μ mol) that had been swelled in THF and the click reaction was allowed to proceed for 14 h. Following the reaction, the resin was washed 5 X DMF, 10 X 10 min with 0.5% sodium diethyldithiocarbamate and 0.5% DIPEA in DMF, 5 X DMF, 5 X CH₂Cl₂, and 5 X MeOH. The peptide was then modified with Sox-Br and cleaved as previously described.

Preparation of phospho-peptides.

In order to determine V_{max} and k_{cat} , preparation of the phosphorylated peptides was necessary, which allows for the conversion of fluorescence units to absolute product formation. Phospho-peptides were produced enzymatically with the kinase of interest. A 50 nmol aliquot of peptide was incubated at 30 °C with 100 ng of the appropriate kinase for 72-120 h in 500 µL of assay buffer containing 50 mM Tris, pH 7.4, 10 mM MgCl₂, 1 mM EGTA, 2 mM DTT, 0.01% Brij-35, and 1 mM ATP. The reaction was monitored by reversed phase HPLC-MS. Following complete conversion of the starting material, the phosphopeptides were purified via HPLC and characterized as described for the unphosphorylated peptides.

Characterization of purified peptides.

The purity of the synthetic peptides was assessed by reversed-phase HPLC and identity was confirmed by ESI-MS.

Table 1. Sequence and molecular formula for each peptide sensor and corresponding phospho-peptide product.

Sensor	Peptide Sequence	Mol. Formula
JNK1/2/3	ERPSRDHLYLPLEP-PEG ₂ -SANLLSP-Csox-PA	$C_{137}H_{213}N_{37}O_{43}S_2$
pJNK1/2/3	ERPSRDHLYLPLEP-PEG ₂ -SANLL(p)SP-Csox-PA	$C_{137}H_{214}N_{37}O_{46}PS_2$
p38α/β	GKKRRKLLLPNSADEIKKIKI-PEG2-Triazole-PEG2-QP-Csox-ASPVV	$C_{179}H_{307}N_{51}O_{49}S_2$
p-p38α/β	GKKRRKLLLPNSADEIKKIKI-PEG2-Triazole-PEG2-QP-Csox-A(p)SPVV	$C_{179}H_{308}N_{51}O_{52}PS_2$
ERK1/2	GLKRVRRQALISSEIPKLQP-PEG2-Triazole-PEG2-VP-Csox-LTPGGRR	$C_{180}H_{307}N_{57}O_{49}S_2$
pERK1/2	GLKRVRRQALISSEIPKLQP-PEG ₂ -Triazole-PEG ₂ -VP-Csox-L(p)TPGGRR	$C_{180}H_{308}N_{57}O_{52}PS_2$

 Table 2. M/S characterization data for each peptide sensor.

Sensor	[M+xH] ^{x+} Calc.	[M+xH] ^{x+} Obsvd.		
JNK1/2/3	[M+2H] ²⁺ : 1565.26	1565.40		
pJNK1/2/3	[M+2H] ²⁺ : 1605.24	1605.53		
p38α/β	[M+3H] ³⁺ : 1340.77	1341.00		
p-p38α/β	[M+3H] ³⁺ : 1367.41	1367.33		
ERK1/2	[M+3H] ³⁺ : 1372.76	1373.80		
pERK1/2	[M+3H] ³⁺ : 1399.41	1400.33		

Determination of kinetic parameters.

Kinetic parameters were derived as described in the Experimental Methods sections and as previously described.¹



Figure 1. Michaelis Menten plots for each MAP kinase activity sensor.

Antibodies. Antibodies used in western blot analysis are as follows: p-JNK: Cell Signaling, #9251 p-ERK: Cell Signaling, #4377 p-p38: Cell Signaling, #9215 Tubulin: Cell Signaling, #3873

MAP Kinase subfamily homology. Sequence alignments were determined within each MAP kinase subfamily using ClustalO. Phylogenic tree for the MAP kinase family was determined by sequence alignment of all MAPK isoforms. The MAP kinase isoforms used are as follows:

Kinase	Accession Num	oer Kinase	Accession N	umber	Kinase	Accession Number
JNK1	P45983.2	p38a	NP_001306.1		ERK1	P27361.4
JNK2	P45984.2	р38β	NP_002742.3	3	ERK2	P28482.3
JNK3	P53779.2	p38ō	NP_002745.1	l	ERK5	Q13164.2
		p38γ	NP_002960.2	2		
			—			
		20			40	
p38alpl	na 🗧 – – – M S QE R P T	FYRQELNKTI	WEVPERYQNL	SPVGSGA	YGS VCA	AFDTKTG 47
p38be	ta – – – M S G P R A G	FYRQELNKTV	WE V P Q R L Q G L	RPVGSGA	YGS VCS	AYDARLR 47
p38del	ta – MSLIRKKG	FYKODVNKTA		THVGSGA		AIDKRSG 48
Consens						
consens	60		80	110304	105 105	100
p38alpl	I RVAVKKISR	PEOSITHAKR				TPARSLE 97
p38be	ta QKVAVKKLSR	PFQSLIHARR	TYRELRLLKH	LKHENVI	GLL DVF	TPATSIE 97
p38del	ta EKVAIKKLSR	PEQSEIFAKR	AYRELLLLKH	MQHENVI	GLL DVF	TPASSLR 98
p38gamn	AKMAIKKLYK	PEQSEL FAKE	AYKELKLIKH		GLL DVE	TRA SLE
Consens	US - KVA - KKLSK	PFQSEI-AKK	-YKELKLLKH	MKHENVI	140 GLL DVF	IPA-SLE
		1			1	
p38alpl	TA EFNDVYLVIH		KCQKLIDDHV			
p38del	ta NEYDEYLVMP	FMQTDLQKIM	GME-FSEEKI	QYLVYQM		HSAGVV 147
p38gamn	na D <mark>FT</mark> DF <mark>YLV</mark> MP	FMGTDLGKLM	KHEK LGEDRI	QFLVYQM	LKG LRY	IHAAGII 150
Consens	us DF-D-YLV-P	- MG - D L N - I -	K C – K L S – – H –	QFLVYQM	L-G LKY	IHSAGII
	160		180 I			200
p38alp	na HRDLKPSNLA	VNEDCELKIL	DFGLARHTDD	EMTGYVA	TRW YRA	PEIMENW 197
p38be	ta HRDLKPSNVA	VNEDCELRIL	DEGLARQADE	EMICYVA	TRW YRA	PEIMENW 197
p38gamn		VNEDCELKIL		EMICYVV	TRW YRA	
Consens	us HRDLKP-NLA	VNEDCELKIL	DFGLAR-AD-	EMTGYV-	TRW YRA	PELNW
		220			240	
p38alpl	na MHYNQTVDIW	SVGCIMAELL	TGRTLFPGTD	H I N <mark>QL</mark> QQ	IMR LTG	TPPAYLI 247
p38be	ta MHYNQTVDIW	SVGCIMAELL	QGKALFPGSD	YIDQLKR	IME VVG	TPSPEVL 247
p38del		SVGCIMAEML		YLDOLTQ	ILK VTC	VPGTEFV 247
Consens					IMK VTC	TPPAFEV
consens	260	SVOCTMAL-L	280	DQLKQ		300
p38alpl						
p38be	ta AKISSEHART	YIQSEPPMPQ	KDLSSIFRGA	NPLAIDL		LDSDQRV 297
p38de	ta QKL <mark>NDK</mark> A <mark>A</mark> KS	Y I QS PQT PR	K D F T Q L F P R A	SPQAADL	LEK MLE	LDVDKRL 297
p38gamn	na QRLQSDEAKN	YMKG PELEK	KDFASILTNA	SPLAVNL	LEK MLV	LDAEQRV 300
Consens	us Q-L-S-EA-N	Y I Q S L P QM P K	KDFASTF-GA	-PLAVDL	240 LEK MLV	LDSD-RV
- 20-1-1						
p38alpl p38ba	TA TAAQALAHAY			VEAKERT		LIYDEVI 346
p38del	ta TAAQALTHPF	EPFRDPEEE	TEAQQPEDDS	LEHEKLT		HIYKEIV 347
p38gamn	na TAGEALAHPY	FESLHDTEDE	PQ-VQKYDDS	FDDVDRT		VTYKEVL 349
Consens	us TAA-ALAH-Y	FEQYHDPEDE	PE-AQPYDDS	F E – – D – T	LDE WK-	LTYKEVL
	360 I					
p38alp	na SEVPPLDQE	EMES 360	D			
p38be		PGSLEIEQ 364	4			
p38gamn	IN SEKPPROLCA	RVSKETPL 36	7			
Consens	us SFKPP	R – S S E – – L				

Figure 2. Sequence alignment for each p38 MAP kinase.



Figure 3. Sequence alignment for each JNK MAP kinase isoform.



Figure 4. Sequence alignment for each ERK MAP kinase.



Figure 5. Phylogenic tree for the MAP kinase family.



Figure 6. Activity of ERK1/2 in HeLa cell lysates following treatment with 1 µM anisomycin. Assays were run in the presence and absence of the p38 inhibitor, SB203580 (500 nM). Values are plotted as fold change from the 0 min control and are representative of triplicate measurements performed in duplicate



Figure 7. Activity of ERK1, p38 α , or a combination of ERK1 and p38 α as detected using the ERK1/2 sensor. p38 α activity can be effectively diminished with the addition of 500 nM SB203580, a selective p38 inhibitor.

Assays with Kinase Inhibitors.

Assays were performed as described in the Experimental Section, but in the presence of selective kinase inhibitors. Inhibitor stocks were made 100X in DMSO. Recombinant kinases or lysates were incubated with inhibitors for 30 min at 30 °C at 1X inhibitor concentration (final concentration of DMSO <1%). Substrate peptides in assay buffer were also incubated with 1X inhibitor prior to kinase/lysate addition. IC_{50} 's were derived by plotting the %Activity of the DMSO (vehicle) control versus inhibitor concentration and curve fit using nonlinear regression analysis was performed in GraphPad Prism to calculate IC_{50} s.



Figure 8. Dose-response curves for each selective MAP kinase subfamily inhibitor in both cell lysates and against recombinant kinase.

References

(1) Luković, E.; González-Vera, J. A.; Imperiali, B. J. Am. Chem. Soc. 2008, 130, 12821.