Electronic Supplementary Material (ESI) for Chemical Communications

Supporting Information

Chemical Activation of MEK1 - a redox trigger for evaulating the effects of phosphorylation

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1. Experimental Section

1.1 Vector Construction and Mutagenesis

MEK1 (mouse) was amplified from pEX_EF1_CFP-MEK1 (ATCC) with the following primers: 5'-ATGATGGGATCCCTTGAAGTTCTGTTCCAAGGGCCCATGCCCAAG AAGAAGCCG-3' (BamHI–PreScission) and 5'ATGATGAAGCTTTCAGATGCTGG CAGCGTG-3' (HindIII) using Deep Vent. The PCR product and the pTrcHis B vector (Invitrogen) were digested sequentially with HindIII and BamHI and then ligated together using T4 DNA ligase. The newly constructed plasmid was sequenced and determined to be missing a nucleotide that was cut out of the pTrcHis B vector. The insertion and all mutations were introduced using the QuikChange Site Directed Mutagenesis Kit (Stratagene) and confirmed by DNA sequencing. The nucleotide was inserted using the following primers: 5'-ATGACG ATAAGGATCCGCTTGAAGTTCT GTTCC-3' and 5'-GGAACAGAACTTCAAGCGGATCCTTATC GTCAT-3'. Mutations: C218,222; C218; C222; and A218 were introduced into the pTrcHis B-MEK1 vector using the following primers:

C218,222 5'-GGGCAGCTAATTGACTGT ATGGCCAACTGCTTCGTGGGCACGAGA-3'and 5'-TCTCGTGCCCACGAAGCA GTTGGCCATACAGTCAATTAGCTGCCC-3'; C218 5'-GTCAGCG GGCAGCTAAT TGACTGTATGGCCAACTCCTTCGTGGGC-3'and 5'-GCCCACGAAGGAGTTGG CCATACAGTCAATTAGCTGCCCGCTGAC; C222 5'-TGACTCTATGGCCAACTGCTTCGTGGGC ACGAGAT-3' and 5'-ATCTCG TGCCCACGAAGCAGTTGGCCATAG AGTCA-3'; A218, 5'-CAGC GGGCAGCTAATTGACGCTATGGCCAACTCCTTCG T-3' and 5'-ACGAAGGAGTTGGCCATAG CGTCAATTAGCTGCCCGCTG-3'.

ERK2 (mouse) was a kind gift from Dr. Pulido.[1] Catalytically inactive ERK2 K52R was obtained using the following primers: 5'-CAAAGTTCGAGTTGCTATCAGGAAAATCAGTCCTTTTGAGC-3'and 5'-GCTCAAAAGGACTGATTTTCCTGATAGCAACTCGAACTTTG-3'. All primers for mutagenesis were designed using Primer X and oligonucleotide synthesis was performed by Integrated DNA Technologies.

1.2 Protein Expression: MEK1 and ERK2

The various protein constructs were expressed according to Gardner *et al* except the temperature was decreased to 22 ^{o}C for 1 hr before induction and induced with 3.25 mM IPTG (OD_{600} ~0.6) according to Ohren *et al*.[2-3] The cells were then incubated for an additional18 hrs at 22 ^{o}C.[3]

1.3 Purification / Preparation of Proteins:

1.3.1 MEK1

The pellet was resuspended in lysis buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol and 0.1% Tween-20) supplemented with 5 μgmL^{-1} leupeptin and 2 μgmL^{-1} aprotinin. The cells were lysed and centrifuged at 10,000g for 30 minutes. The supernatant was then subject to Ni^{+2}-NTA agarose (Qiagen) affinity chromatography. The protein was allowed to bind for 1 hr at 4 ^{o}C and the resin was then washed with lysis buffer followed by wash buffer (20 mM sodium phosphate, pH 7.7, 0.5 M NaCl, 5 mM imidazole, 1 mM β-mercaptoethanol and 0.1% Tween-20). The protein was eluted with 20 mM sodium phosphate, pH 7.7, 300 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol and 0.1% Tween-20. The fractions that contained MEK1 were subject to gel filtration using a Superdex 200 column (GE Healthcare) and eluted in MEK1 storage buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 1 mM benzamidine, 0.025% Triton X-100, 5% glycerol and 0.03% brij-35) supplemented with 1 mM β-mercaptoethanol.

1.3.2 ERK2

The pellet was resuspended in lysis buffer (PBS, 1% Triton X-100 and 5 mM DTT) supplemented with 5 μgmL^{-1} leupeptin and 2 μgmL^{-1} aprotinin. The cells were lysed and centrifuged at 10,000g for 30 minutes. The protein was purified using Glutathione Sepharose resin (GE Healthcare) and was allowed to bind for 30 minutes at RT. The resin was washed with lysis buffer and the protein was eluted in 50 mM Tris, pH 8.0, 1% Triton X-100, 5 mM DTT and 10 mM reduced glutathione. The protein was then dialyzed into 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 1 mM benzamidine, 50% glycerol and 0.03% brij-35.

1.3.3 Raf-1

Active human Raf-1(Upstate) was dialyzed into 50 mM Tris, pH 7.5, 150 mM NaCl, 0.1 mM EGTA, 1 mM benzamidine, 270 mM sucrose and 0.03% brij-35.

1.4 Phosphorylation of MEK1

Phosphorylation of MEK1 and enzyme activity assays were modeled after the Raf-1 Kinase Cascade Assay Kit (Upstate). MEK1 (~130 nM) was incubated at 30 ^{o}C for 1 hr with 8.3 mM MOPS, pH 7.2, 10.4 mM β-glycerophosphate, 2.1 mM EGTA, 0.4 mM sodium orthovanadate, 62 μM ATP, 9.3 mM $MgCl_2$ and 0.5 nM Raf-1 maintaining 5% glycerol and 0.03% brij-35. MEK1 was then subject to gel filtration using a Superdex 200 column and eluted in MEK1 storage buffer or dialyzed into MEK1 storage buffer supplemented with 1 mM β-mercaptoethanol and then subject to sulfonation.

1.5 Sulfonation conditions

MEK1 was incubated with ATP (27 mM) and $MgCl_2$ (67 mM) for 5 minutes at RT to protect vital cysteine residues from sulfonation.[4-6] The reaction was initiated by adding sodium tetrathionate (19.4 mM) and sodium sulfite (88 mM) while maintaining 5% glycerol and 0.03% brij-35. The reaction was allowed to proceed overnight at RT and then subject to gel filtration using a Superdex 200 column and eluted in MEK1 storage buffer. The concentration of MEK1 was determined by the MEK1 Total ELISA kit (Invitrogen). *Note the addition of 5% glycerol[7-9] and 0.03% brij-35 were required in the sulfonation reaction to prevent a majority of the protein from aggregating and the remaining aggregates were then removed by gel filtation.*

1.6 Enzyme Activity Assay

MEK1 (2.9 or 5.8 nM) was incubated with or without DTT (110 mM) for 20 minutes at RT. The activity was then evaluated by incubating MEK1 (0.9 or 1.8 nM) at 30 ^{o}C for 30 minutes with 12.4 mM MOPS, pH 7.2, 15.5 mM β-glycerophosphate, 3.1 mM EGTA, 0.6 mM sodium orthovanadate, 14 mM $MgCl_2$, 91 μM ATP, 94 $nCi\mu L^{-1}$ [γ-^{32}P] ATP and 20 μM ERK2 K52R. Reactions were performed in triplicate and the total reaction volume was 32 μL. Phosphoric acid (0.6%) was used to terminate the reaction and the sample was loaded onto a P81 phosphocellulose square and washed three times with 5 mL of 0.75%

phosphoric acid for 10 minutes and then washed with 5 mL of acetone for 5 minutes. The CPM of the samples were determined using the liquid scintillation analyzer Tri-Carb 2900 TR (PerkinElmer) and the pmoles of phosphate transfered were calculated using equation 1. To evaluate nonspecific binding of [γ-^{32}P] ATP, a negative control reaction was run that omitted MEK1.

$$\frac{[(\text{CPM sample} - \text{CPM negative control}) \times \text{pmoles ATP reaction}]}{\text{CPM total counts available}} \quad \textbf{(1)}$$

1.7 Enzyme Kinetics

MEK1 (0.65 nM) was incubated at 30 ^{o}C for 30 minutes with 12.4 mM MOPS, pH 7.2, 15.5 mM β-glycerophosphate, 3.1 mM EGTA, 0.6 mM sodium orthovanadate, 14 mM $MgCl_2$, 94 nCiμL^{-1} [γ-^{32}P] ATP, 20 μM ERK2 K52R and varying concentrations of ATP (0.57 - 91.5 μM). Reactions were performed in triplicate and the total reaction volume was 21.2 μL. Reactions were terminated with dilute phosphoric acid and treated as previously stated.

1.8 Dot Blot

MEK1 was incubated with or without DTT (100 mM) for 20 minutes at RT. MEK1 (0.3 ng) was bound to the nitrocellulose membrane using the Bio-Dot microfiltration apparatus (Bio-Rad). The membrane was washed and removed from the apparatus prior to blocking. The membrane was probed with anti-MEK1 [pSpS218/222] (Biosource) (1:1,000) followed by anti-rabbit HRP (Cell Signaling) (1:1,000). Supersignal west pico chemiluminescent substrate (Thermo Scientific) was used for detection and exposed for 1 hr. The film was developed and then fixed.

2. Supplemental Figures

Initially to investigate whether site-specific sulfonation of cysteine can mimic phosphorylation in MEK1, the enzymatic activity of various C218,222 and Wt MEK1 constructs were evaluated. Figure S1 shows that $C218,222^{Sulfo}$ and Wt^{Sulfo} had 19% and 8.5% of $Wt^{Raf\text{-}Sulfo}$ enzymatic activity. In the presence of the reducing agent dithiothreitol (DTT) the enzymatic activity of $C218,222^{Sulfo}$ was completely abolished, whereas most of the enzymatic activity for $Wt^{Raf\text{-}Sulfo}$ and Wt^{Sulfo} were retained. These results indicate that $C218,222^{Sulfo}$ was activated by sulfonation of cysteine residue(s), potentially 218 and or 222; whereas Wt^{Sulfo} is activated by a completely different mechanism. Since it has been previously established that MEK1 could be activated through the introduction of proline residues in its N-terminus[10] it is possible that activation of Wt MEK1 may involve sulfonation of at least one of the six potential cysteine residues, which in turn may become less accessible to reduction due to conformational changes.

Huang *et al* demonstrated that the introduction of a single acidic residue only partially activates MEK1, whereas the introduction of both acidic residues leads to a synergistic effect that results in complete activation.[11] Thus, if there was a problem modifying one cysteine residue or sulfonation of cysteine at one residue was not an effective surrogate the enzymatic activity would be significantly affected. To interrogate the sulfonation of each cysteine, individual cysteine mutant constructs were generated. The MEK1 constructs were first phosphorylated with Raf-1 and then subject to sulfonation. The ability of $C218^{Raf\text{-}Sulfo}$ and $C222^{Raf\text{-}Sulfo}$ to phosphorylate ERK2 was then evaluated by SDS-PAGE and the relative activities were determined by autoradiography. Since $C222^{Raf\text{-}Sulfo}$ only exhibited 14% of $C218^{Raf\text{-}Sulfo}$ enzymatic activity, $C218^{Raf\text{-}Sulfo}$ became the main focus of this study.

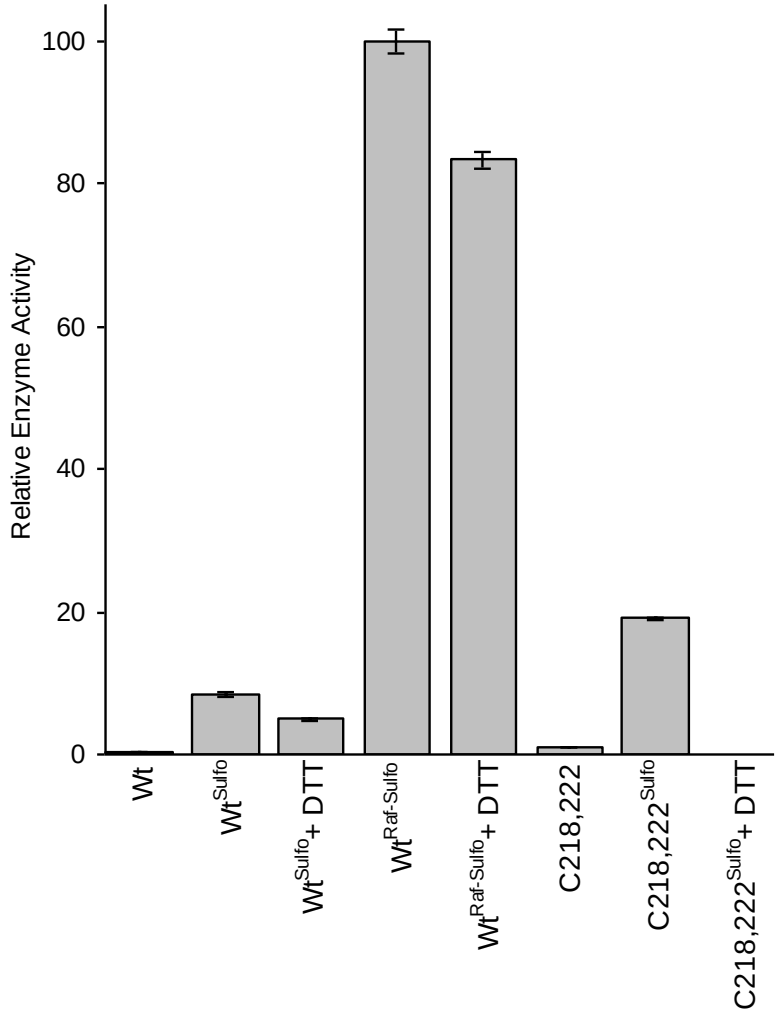

Figure S1. Activation of MEK1 mutant C218,222 by sulfonation of cysteine. MEK1 constructs (0.9 nM) were evaluated for their ability to phosphorylate ERK2 K52R. Reactions were performed in triplicate and the average is reported +/- SD.

3. References

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