ELUCIDATING THE EFFECT OF THE MK-STYX KIM DOMAIN MUTATION ON DOCKING TO MAPK

Zoe Zmuda
Department of Biology, College of William & Mary
Advisor: Shantá D. Hinton, Ph.D.

Abstract

MK-STYX [MAPK (mitogen-activated protein kinase) phosphoserine/threonine/tyrosine-binding protein] is a catalytically inactive member of the MAPK phosphatase (MKP) family containing mutations in both its dual-specificity phosphatase (DUSP) domain and kinase interaction motif (KIM). Active homologs, MKP-1 and MKP-3, contain consecutive arginine residues in the KIM required for MAPK docking, whereas MK-STYX contains only a single arginine. To test whether restoring these residues repairs docking, MK-STYX KIM constructs (RR, RRR, V53R) were co-expressed with ERK1/2 in HEK-293 cells and analyzed by co-immunoprecipitation and SDS-PAGE. All constructs exhibited minimal ERK1/2 binding, indicating that restoration of canonical KIM arginines alone is insufficient to re-establish complete docking. These findings refine our understanding of MK-STYX structure and function relationships and inform future investigation of its role in MAPK-associated signaling pathways implicated in cancer and metabolic disease.

Introduction

Cells are constantly communicating through hundreds of intricate cell signaling pathways, many of which use phosphate molecules. When added to a protein (phosphorylation) or removed from a protein (dephosphorylation), these phosphates cause conformational changes to the bound protein, often resulting in the activation or deactivation of key biological processes such as ATP production and metabolism, as well as homeostasis[1]. Kinases and phosphatases are responsible for the addition and removal of phosphate groups from proteins, respectively.

In the last 50 years, scientists have discovered proteins conveniently named "pseudoenzymes," as they are homologous to their active enzyme counterparts, but lack certain amino acids needed for catalytic activity[2]. However, the last decade has proven pseudoenzymes as important cell signaling regulators[2,3].

MK-STYX is a pseudoenzyme (pseudophosphatase) with mutations in its DUSP domain, and the KIM sequence within the CH2 domain[3,4]. To regulate cell signaling processes, MKPs [MAPK (mitogen-activated protein kinase) phosphatases] bind to and dephosphorylate MAPKs/ERKs (extracellular signal regulated kinases), the target kinases[3–5]. For this process to occur, the MKP's CH2 domain must bind to the phosphorylated kinase, inducing a conformational change and allowing the DUSP domain to remove the dual-phosphate groups from the bound kinase (Figure 1)[3,4].

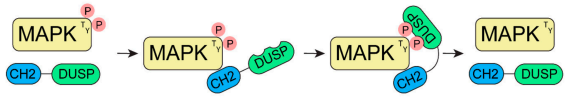

Figure 1: Canonical MAPK dephosphorylation mechanism. The CH2 domain of a MKP binds to the negatively charged docking groove of a MAPK, inducing a conformational change that positions the catalytic DUSP domain for activity. The DUSP domain dephosphorylates both the threonine and tyrosine residues within the MAPK activation loop, leading to inactivation of the MAPK signaling cascade[4].

MKP DUSP domains have a highly conserved active site motif, consisting of histidine and cysteine, followed by five amino acids, then an arginine (HCX_5R)[3,4]. The MK-STYX DUSP domain lacks the

imperative histidine and cysteine amino acids, inhibiting dephosphorylation[3] (Figure 2). In a similar manner, MKP KIM sequences contain consecutive arginine amino acids. However, MK-STYX's KIM domain has a single arginine (Figure 3)[3].

PTP Member	Active Site Motif
DUSP-1/MKP-1	**H C** Q A G I S **R**
DUSP-6/MKP-3	**H C** L A G I S **R**
DUSP-24/MK-STYX	F S T Q G I S **R**
Consensus	**H C** X X X X X **R**

Figure 2: Active site motif comparison of MKP homologs. Sequence alignment showing the highly conserved PTP catalytic motif, HCX_5R. In MK-STYX, phenylalanine (F) and serine (S) substitutions replace the canonical histidine and cysteine residues, inhibiting catalytic phosphatase activity[3].

KIM Sequence	48	49	50	51	52	53	54	55	56	57
DUSP-1/MKP-1	T	I	V	R	R	R	A	K	-	G
DUSP-6/MKP-3	G	I	M	L	R	R	L	Q	K	G
DUSP-24/MK-STYX	I	T	A	L	R	V	K	K	K	N
Consensus	X	I	X	L	R	R	X	K	K	G

Figure 3: KIM sequence comparison of MKP homologs. Sequence alignment of MKP-1, MKP-3, and MK-STYX highlights the loss of consecutive arginine residues in the MK-STYX KIM domain, a feature required for MAPK docking in active MKPs[3].

Although the specific KIM sequence mutation that prevents binding in MK-STYX is unknown, it is theorized that the lack of consecutive arginines is a key component[3–5]. Arginines have positively charged side chains which bind to the negative amino acid residues of the MAPK/ERK1/2 binding pocket[3,5]. With that in mind, it can be hypothesized that MKP's consecutive arginines in the KIM domain create a stronger positive charge than MK-STYX's single arginine, resulting in a difference in KIM folding and, as a result, the lack of kinase binding[4].

Understanding the source of binding incapability between MK-STYX and MAPK will create more opportunities for MK-STYX to be used in developing therapeutic treatments and other critical research projects.

Objectives

To evaluate the binding of MAPK to various mutated MK-STYX constructs. Also, to test whether the loss of consecutive arginine residues in the KIM domain induces a conformational change that inhibits docking.

Methodology

The interaction (or lack thereof) between MK-STYX or MKP and ERK1/2 can be determined through co-immunoprecipitation (co-IP) and immunoblotting.

Transformation

Bacterial transformation *Escherichia coli* DH5α competent cells (Agilent) were used. Plasmid DNA was introduced into competent cells via heat shock, followed by incubation to facilitate DNA uptake. Transformed cells were plated on agar plates containing either ampicillin (Fisher Scientific) or kanamycin (Fisher Scientific), depending on plasmid antibiotic resistance, and were incubated overnight at 37°C in a dry incubator. The pCMV (GenScript) and pEGFP (Addgene) plasmids were grown on kanamycin agar plates, and the pSG5 plasmids, which were kindly provided as a gift from Dr. Nicholas K. Tonks, were grown on ampicillin agar plates.

Plasmid Preparation

To prepare plasmids for transfection, bacterial colonies were obtained from the transformation agar plates and were cultured overnight at 37°C and 250 RPM in Luria broth supplemented with the appropriate antibiotic based on plasmid resistance. Cultures were centrifuged the following day to pellet bacterial cells, and plasmid DNA was isolated using the ZymoPure Plasmid Midiprep Kit according to the manufacturer's protocol.

Co-transfection

Transfection was performed to introduce MKP and MK-STYX constructs into HEK-293 cells at approximately 70% confluency. MKP-1 and MKP-3 served as the positive controls in this experiment due to their known phosphatase activity[5], while empty pCMV vector was used as the negative control. The pCMV-MK-STYX$_{wild\text{-}type}$ construct represented the native MK-STYX protein without laboratory-introduced mutations. Mutant MK-STYX constructs included pCMV-MK-STYX$_{RR}$, containing two consecutive arginines within the KIM at positions 51 (L51R) and 52; pCMV-MK-STYX$_{RRR}$, containing three consecutive arginines at positions 51 (L51R), 52, and 53 (V53R); and pCMV-MK-STYX$_{V53R}$, containing two consecutive arginines at positions 52 and 53 (V53R). The pCMV-MK-STYX$_{active\ mutant}$ represented a laboratory-engineered form of MK-STYX.

Cells were transfected with pSG5-MKP-1, pSG5-MKP-3, pCMV, pCMV-MK-STYX$_{wild\text{-}type}$, pCMV-MK-STYX$_{RR}$, pCMV-MK-STYX$_{RRR}$, pCMV-MK-STYX$_{V53R}$, pCMV-MK-STYX$_{active\ mutant}$ using Lipofectamine 2000 (Thermo Fisher) in Opti-MEM reduced-serum medium (Gibco). Cells were co-transfected with pEGFP-ERK1, and complexes were prepared using 8 µL Lipofectamine with 2 µg plasmid DNA and 2 µg pEGFP-ERK1. Following transfection, cells were returned to DMEM supplemented with 10% FBS and incubated at 37°C with 5% CO_2 overnight.

Lysis

Cells were lysed to generate protein samples for downstream analysis by Western blotting. Transfected cells were washed with 1X Dulbecco's Phosphate-Buffered Saline (D-PBS) and lysed using NP-40 lysis buffer (HEPES (Fisher Scientific), NaCl, 100% glycerol (Fisher Scientific), NP-40 alternative (Calbiochem)). Lysis buffer was supplemented with protease inhibitor cocktail (Sigma-Aldrich) and PhosSTOP phosphatase inhibitor tablets (Sigma-Aldrich), used to prevent protein degradation and inhibit dephosphorylation, respectively.

Lysate Sample Preparation

Lysates were processed prior to electrophoresis to ensure uniform protein loading. Protein concentration was determined using the Pierce 660 nm Protein Assay (Thermo Scientific) and measured using a NanoDrop spectrophotometer (Thermo Scientific). Samples were diluted with NP-40 lysis buffer to achieve equal amounts of protein per sample, in µg. 5 µL of 6X sample loading buffer were added to each sample to visualize protein migration. Additionally, 2 µL of dithiothreitol (DTT) (GE HealthCare) was included to prevent disulfide bond formation and ensure protein denaturization.

Co-immunoprecipitation (co-IP)

Co-IP assays were performed to assess protein-protein interactions between ERK1/2 and MKP/MK-STYX constructs. Protein lysates were pre-cleared using Protein G Sepharose beads (Cytiva) to reduce non-specific binding. Following pre-clearing, lysates were incubated with either anti-FLAG or anti-Myc primary antibodies, depending on the epitope tag of the expressed construct. Protein G Sepharose beads were then added to capture antibody-protein complexes.

Samples were incubated at 4ºC for 1 hour with gentle mixing to allow immune complex formation. Beads were pelleted by centrifugation, and supernatants were discarded. Bead-bound complexes were washed four times with NP-40 lysis buffer to remove non-specifically bound proteins. After the final wash, beads were resuspended in 30 µL of 6X sample loading buffer supplemented with 2 µL of DTT. Samples

were heated at 100ºC for 4 minutes and prepared for SDS-PAGE, followed by Western blot analysis.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate and detect proteins by molecular weight (kDa). 10 well gels were cast using 0.75 mm plates (Bio-Rad) with a final acrylamide concentration of 10%. During electrophoresis, lower molecular weight proteins moved further through the gel, while higher molecular weight proteins remained closer to the top.

The wells were loaded in the following order: Precision Plus Protein Kaleidoscope ladder (Bio-Rad), pSG5-MKP-1, pSG5-MKP-3, Precision Plus Protein Kaleidoscope ladder, pCMV, pCMV-MK-STYX$_{wild\text{-}type}$, pCMV-MK-STYX$_{RR}$, pCMV-MK-STYX$_{RRR}$, pCMV-MK-STYX$_{V53R}$, pCMV-MK-STYX$_{active\ mutant}$. The protein ladder was loaded twice to provide molecular weight references for both plasmid vectors following membrane cutting, as the vectors contained different epitope tags.

Gels were submerged in SDS-PAGE running buffer and run at 200 V for approximately 45 minutes using a Bio-Rad power supply. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad) using Bio-Rad's Trans-Blot semi-dry transfer system and Trans-Blot Turbo Transfer Pack.

Immunoblotting

Prior to antibody probing, PVDF membranes were blocked for 1 hour in 5% non-fat dry milk (Apex) prepared in 1X Tris-buffered saline with Tween (TBST). Membranes were washed three times for 5 minutes with 1X TBST between blocking, antibody incubations, and mild stripping steps.

Both primary and secondary antibodies were diluted 1:1000 in 5% non-fat dry milk prepared in 1X TBST. Primary antibodies were incubated overnight at 4ºC, followed by incubation with secondary antibodies for 1 hour at room temperature. Membranes were developed for 5 minutes using enhanced chemiluminescence (ECL) Prime Western Blotting Detection Reagents (Cytiva) and imaged using an iBright 1500 imaging system (Invitrogen). Membranes were stripped after imaging according to Abcam's Mild Stripping Protocol.

Total ERK was detected using the ERK1/2 (C-9) primary antibody (Santa Cruz Biotechnology). For lysate Western blots, anti-mouse IgG (Invitrogen) was used as the secondary antibody. For IP membranes, Mouse TrueBlot secondary antibody (Rockland) was used to avoid interference from IgG heavy and light chains.

FLAG-tagged pCMV proteins were detected using anti-FLAG M2 primary antibody (Millipore Sigma), while Myc-tagged pSG5 proteins were detected using an anti-Myc primary antibody (Thermo Fisher). Membranes were cut prior to anti-FLAG and anti-Myc primary incubation to allow simultaneous probing of proteins from different vectors. Anti-mouse IgG and anti-rabbit IgG were used as secondary antibodies for FLAG and Myc detection, respectively.

β-tubulin (Invitrogen) was used as a loading control, ensuring equal protein loading across samples. Anti-rabbit IgG was used as the secondary antibody.

Results

MK-STYX KIM mutants with restored consecutive arginine sequences were utilized to test for restoration of ERK1/2 docking. Figure 4 shows the KIM sequence alignments of pCMV-MK-STYX$_{wild\text{-}type}$, as well as the MK-STYX constructs used, pCMV-MK-STYX$_{RR}$, pCMV-MK-STYX$_{RRR}$, and pCMV-MK-STYX$_{V53R}$.

Figure 4: Engineered KIM sequence variants of MK-STYX. Wild-type MK-STYX and KIM mutants (RR, RRR, V53R) highlighting restoration of consecutive arginine residues. Constructs were co-expressed with ERK1/2 and evaluated for interaction by co-immunoprecipitation.

Figure 5 shows the immunoprecipitation membrane. The second and third lanes in Figure 5B show bound active MKPs to ERK1/2, acting as the positive control. Lanes seven, eight, and nine, which contain the MK-STYX constructs, demonstrate slightly restored ERK1/2 docking, but evidently, binding is not restored to the full effect of the active MKP homologs. However, there is variation in the interactions of ERK1/2 to the MK-STYX constructs. It appears that ERK1/2 interacts with MK-STYX$_{RRR}$ more than the other variants, MK-STYX $_{RR}$ and MK-STYX$_{V53R}$.

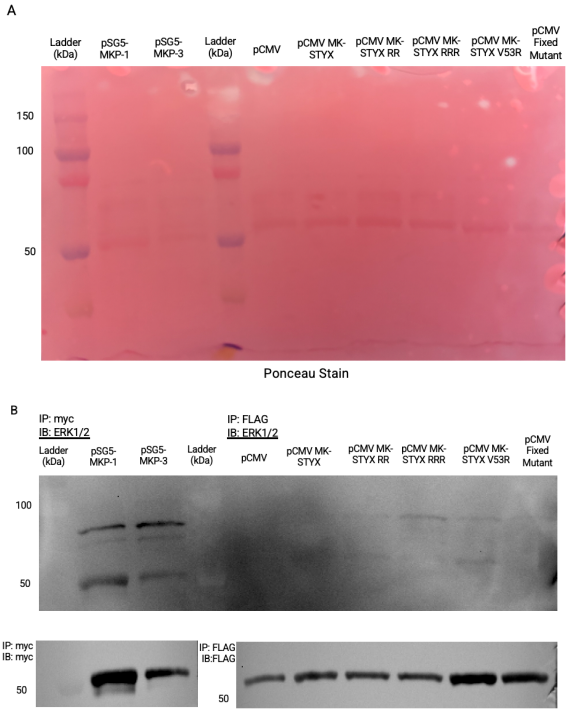

Figure 5: MK-STYX KIM mutants fail to fully restore ERK1/2 binding. (A) Ponceau stain of immunoprecipitated samples showing IgG heavy and light chains. (B) ERK1/2 detected with TrueBlot secondary antibody. Strong ERK1/2 binding seen with MKP-1 and MKP-3, while MK-STYX KIM mutants (RR, RRR, V53R) exhibit minimal binding. Blots probed with anti-Myc or anti-FLAG antibody confirm successful immunoprecipitation.

Figure 6 shows the Western blot membrane containing the whole-cell lysates, which demonstrate the presence of each protein, including ERK1/2, MK-STYX, and the active MKPs.

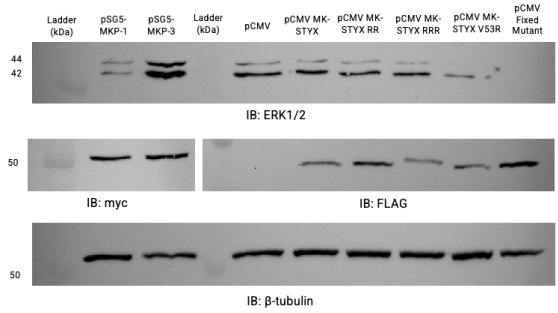

Figure 6: Validation of protein expression in whole-cell lysates. Whole-cell lysates were analyzed by immunoblot prior to immunoprecipitation. ERK1/2 (44 and 42 kDa) confirms kinase presence across samples. Anti-Myc and anti-FLAG probing verifies expression of MKP and MK-STYX constructs. β-tubulin (55 kDa) serves as a loading control.

Discussion

The pseudophosphatase MK-STYX is unable to both bind and dephosphorylate MAPK/ERK, a critical part of cell signaling regulation. While the cause of the inability to dephosphorylate has been researched[6,7], the cause of the loss of kinase binding has not. It is hypothesized that the lack of consecutive arginine residues in MK-STYX's KIM domain may impede docking to MAPKs.

Based on the data obtained, restoration of consecutive arginines in the MK-STYX KIM minimally restores ERK1/2 binding, suggesting there are likely broader structural features dictating MK-STYX-MAPK interactions. This data also suggests that, rather than regulation through dephosphorylation, MK-STYX is allosterically regulating signaling pathways.

Through better understanding of the KIM domain mutation, we will be able to view the MK-STYX mechanism as a whole, opening up new opportunities for its applications in research, including space research studies.

Microgravity environments free cells from many gravitational and mechanical stressors, ultimately resulting in misshapen

cell structures and dysregulated cell processes[8,9]. For example, the cytoskeleton becomes misshapen after exposed to microgravity, potentially causing "leaky" ion channels, and drastically impacting biological processes[9]. Due to a weakened immunity, astronauts on missions also face risk of hypoxia, low oxygen levels in tissues, which could result in long-term heart or brain damage[9].

Scientists are still determining why microgravity mechanistically affects cells in this way; a deeper understanding of MK-STYX could benefit these studies. MK-STYX has been found to inhibit formation of stress granules, protective mechanisms in cells used to combat stressors such as radiation or hypoxia[7]. This along with other MK-STYX regulatory features such as inducing and enhancing neurons (brain cells)[10] present a significant opportunity for MK-STYX to become part of some programs studying gravitational effects on the body, such as NASA's Human Research and Space Biology Programs[8,11]. MK-STYX's ability to regulate cellular responses could lead to key advancements in understanding cell biology in extreme environments, benefitting both space missions and overall human health.

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