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Structure of MST2 SARAH domain provides insights into its interaction with RAPL

Guoguang Liu [a,1], Zhubing Shi [b,1], Shi Jiao [b,1], Zhenzhen Zhang [a], Wenjia Wang [b], Cuicui Chen [b], Qiao Hao [b], Meng Zhang [b], Miao Feng [b], Liang Xu [b], Zhen Zhang [b], Zhaocai Zhou [b,*], Min Zhang [a,*]

[a] School of Life Sciences, Anhui University, Hefei, Anhui 230039, China
[b] State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT

The STE20 kinases MST1 and MST2 are key players in mammalian Hippo pathway. The SARAH domains of MST1/2 act as a platform to mediate homodimerization and hetero-interaction with a range of adaptors including RASSFs and Salvador, which also possess SARAH domains. Here, we determined the crystal structure of human MST2 SARAH domain, which forms an antiparallel homodimeric coiled coil. Structural comparison indicates that SARAH domains of different proteins may utilize a shared dimerization module to form homodimer or heterodimer. Structure-guided mutational study identified specific interface residues critical for MST2 homodimerization. MST2 mutations disrupting its homodimerization also impaired its hetero-interaction with RAPL (also named RASSF5 and NORE1), which is mediated by their SARAH domains. Further biochemical and cellular assays indicated that SARAH domain-mediated homodimerization and hetero-interaction with RAPL are required for full activation of MST2 and therefore apoptotic functions in T cells.

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1. Introduction

The STE20 like kinases MST1 and MST2 are core components of mammalian Hippo signaling pathway, which plays a fundamental role in organ size control and tumor suppression (Avruch et al., 2006; O'Neill and Kolch, 2005; O'Neill et al., 2004; Song et al., 2010). MST1/2 in conjunction with an adaptor protein Salvador phosphorylates and activates LATS1/2 kinases, which in turn associates with adaptor protein MOB1, to phosphorylate and prevent nuclear translocation of the downstream transcription coactivator Yes-associated protein (YAP), and thereby inhibit cell proliferation and promote apoptosis (Oka et al., 2008; Wu et al., 2003).

MST1 and MST2 share high sequence homology with identical N-terminal kinase domains for catalysis and distinct C-terminal SARAH (Salvador, RASSF and Hpo homology) domains for homodimerization or hetero-interaction with Salvador and RASSF (Ras association domain family) proteins (Fig. 1A) (Pfeifer et al., 2010; Scheel and Hofmann, 2003). Functional redundancy between MST1 and MST2 has been evidenced by animal studies showing that MST1/2 double knockout causes embryonic lethality while single knockout of either MST1 or MST2 does not impair organ development (Oh et al., 2009; Zhou et al., 2009). Ablation of both MST1 and MST2 caused liver tumorigenesis through largely abolishing YAP phosphorylation and increasing YAP nuclear localization (Zhao et al., 2009). On the other hand, despite of high degree sequence homology and functional redundancy, MST1 and MST2 displayed differential regulatory properties in certain aspects such as susceptibility to protein phosphatases (Deng et al., 2003). The structure of MST1 SARAH domain has been determined by NMR, showing an antiparallel dimeric conformation (Hwang et al., 2007), yet the structure of MST2 SARAH domain has not been studied in detail.

In addition to their critical functions in development, MST1/2 kinases have been implicated in immune regulation including T cell proliferation, activation, and homeostasis, lymphocyte chemotaxis and trafficking, as well as inflammatory responses (Dong et al., 2009; Katagiri et al., 2009; Mou et al., 2012; Yun et al., 2011; Zhou et al., 2008). Crosstalk of MST1/2 with Akt signaling may antagonize Akt1 activity and therefore modulate T cell costimulation and NF-κB target gene transcription (Cinar et al., 2007). In MST1 and MST2 double knockout mice, mature T cells could not efficiently migrate from thymus to the circulation and secondary lymphoid organs, indicating that MST1/2 may control lymphocyte trafficking

* Corresponding authors. Address: State Key Laboratory of Cell Biology, Institute of Biochemistry and Cell Biology, 320 Yueyang Road, Shanghai 200031, China. Fax: +86 21 54921291 (Z. Zhou).
E-mail addresses: zczhou@sibcb.ac.cn (Z. Zhou), zhmin07@ustc.edu (M. Zhang).
[1] These authors contribute equally to this work.

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and migration, which is important for efficient immunosurveillance and effective immune responses (Katagiri et al., 2009; Mou et al., 2012). Consistently, mice lacking MST1 exhibit a variety of T cell abnormalities (Mou et al., 2012); while patients lacking MST1 displayed primary immunodeficiency like features (Abdollahpour et al., 2012).

Recent studies suggest that a group of RASSF proteins including RASSF1-6 may interact with MST1/2 to differentially regulate Hippo signaling (Hwang et al., 2007; Ikeda et al., 2009; Pfeifer et al., 2010; Romano et al., 2010; Vichalkovski et al., 2008). The specific mechanism of RASSF-mediated differential regulation of MST1/2, and its functional role in immune regulation remains largely unknown. The regulator for cell adhesion and polarization enriched in lymphoid tissues (RAPL), also named RASSF5 and NORE1, has been found to associate with MST1 to induce cell polarity and adhesion of lymphocytes (Katagiri et al., 2006). RAPL is an effector for small GTPase Rap1 that is essential for lymphocyte trafficking. During TCR stimulated induction of polarized morphology and clustering of the integrin LFA-1 in T cells, RAPL can associate with MST1 to alter its cellular localization and kinase activity (Katagiri et al., 2009). Meanwhile, animal study suggested that RAPL-MST1 complex negatively regulate naïve T cell proliferation and that MST1 is required for the maintenance of RAPL level in lymphoid cells (Zhou et al., 2008).

It is generally understood that RAPL may associate with MST1/2 through their SARAH domains. Here, we performed structural and biochemical studies of MST2-RAPL signaling. Our crystallographic analysis of MST2 SARAH domain revealed an antiparallel conformation similar to that of MST1 with local variations. SARAH domains are sufficient for MST2-RAPL stable association. Mutations of the SARAH domain interface impaired MST2 homodimerization and interaction with RAPL, preventing full activation of MST2. RAPL stimulates the kinase activity of MST2 *in vitro*, and promotes its apoptotic function *in vivo*. Mutation disrupting MST2 association with RAPL abrogated their cooperative function in T cell apoptosis.

2. Materials and methods

2.1. Cloning, protein expression and purification

Human MST2 SARAH domain (amino acids 436–484) were cloned into HT-pET-28a and expressed in *Escherichia coli* (*E. coli*) BL21(DE3) codon plus cells. The expression of proteins was induced by 0.5 mM isopropyl β-D-thiogalactopyranoside in Luria-Bertani medium. After cultured for 12 h at 16 °C, bacterial cells were harvested by centrifugation and suspended with lysis buffer (20 mM Hepes, 500 mM NaCl, 5% glycerol, 1 mM DTT and 20 mM imidazole, pH 7.5), and then lysed. The soluble cell lysate was fractionated after centrifuged at 18,000*g* for 40 min. The protein was purified with affinity column pre-charged with Ni^{2+}. The proteins was eluted with elution buffer (20 mM Hepes, 500 mM NaCl, 5% glycerol, 1 mM DTT and 300 mM imidazole, pH 7.5) and digested by TEV protease to remove the N-terminal His-tag. Then the proteins were concentrated and loaded on HiLoad 16/60 Superdex 200 column in 20 mM Hepes, 100 mM NaCl, 1 mM DTT, pH 7.5. The purity of proteins was monitored by SDS–PAGE.

Selenomethionine (Se-Met)-substituted MST2 SARAH domain was purified as described above, except that *E. coli* cells were cultured in M9 minimal medium containing amino-acid supplement (lysine, phenylalanine, threonine to final concentration of 100 mg/l, isoleucine, leucine, valine to 50 mg/l, and L-Se-Met to 60 mg/l).

Other MST2 mutants and mouse RAPL fragments were constructed and purified by similar procedures described above. For cell-based assays, Flag-tagged MST2, Myc-tagged MST2 and Flag-tagged human RAPL were subcloned into BamHI/XhoI sites of pcDNA3.1.

2.2. Crystallization, structure determination and refinement

The native or Se-Met-substituted MST2 SARAH domain was concentrated to 16 mg/ml and crystallized by sitting drop vapor diffusion method at 16 °C, in a drop with 1 μl protein solution and 1 μl reservoir solution. The MST2 SARAH domain was crystallized in 0.4 M calcium chloride dihydrate, 0.1 M sodium acetate trihydrate pH 4.6, 5% v/v 2-propanol.

Diffraction date were collected from a flash cooled crystal at 100 K on beamline BL17U of Shanghai Synchrotron Radiation facility (SSRF), China, and processed in HKL2000 (Otwinowski and Minor, 1997). The structure of MST2 SARAH domain was solved using single-wavelength anomalous diffraction method from a Se-Met derivatized with program AutoSol in Phenix (Adams et al., 2010). The model building was performed in Coot (Emsley et al., 2010) and the structure was refined using REFMAC5 (CCP4, 1994; Murshudov et al., 2011).

2.3. Immunoprecipitation

HEK293T cells were maintained in DMEM medium with 5% (vol/vol) fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) in 5% CO_2/95% humidified air at 37 °C at a density of 10^6 cells per ml. Cells were then transiently with expression vectors encoding Flag-tagged wild-type MST2 and Myc-tagged MST2 mutants. Cell extracts were prepared in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 0.5 mg/ml BSA and protease inhibitor cocktail), and incubated with anti-Flag antibody (sigma) for 4 h at 4 °C followed by overnight incubation with Protein A/G agarose (Santa Cruz). The immunoprecipitates were washed three times in the lysis buffer before loading onto SDS–PAGE gels. The samples separated by SDS–PAGE is blotted and developed with anti-Myc antibodies.

2.4. Cross-linking experiment

Purified 1 mg/ml wild-type or mutant MST2 was incubated with 0 and 0.005% (vol/vol) glutaraldehyde (GA) in conjugation buffer (20 mM Hepes, 100 mM NaCl, 1 mM DTT, pH 7.5) at room temperature for 1 h. After the reaction was quenched with 50 mM Tris–Cl pH 8.0 for 30 min, the cross-linked samples were analyzed on a 8% SDS–PAGE.

2.5. Kinase assay

The activity of purified wild-type or mutant MST2 was assayed by using myelin basic protein (MBP) as substrate. Kinase activity of wild-type or mutant MST2 was measured in a total assay volume of 40 μl consisting of 50 mM Tris–HCl pH 7.5, 0.1 mM EGTA, 1 mM DTT, 10 mM magnesium acetate, 0.1 mM [γ-^{32}P]ATP (200 c.p.m./pmol) and 1 mg/ml MBP, with 18.5 μM MST2 and 92.5 μM RAPL. The assays were carried out at 30 °C and were terminated after 20 min by spotting the 40 μl reaction mixture onto P81 membranes. The membranes were washed in phosphoric acid for 3 times, and the incorporated radioactivity was measured by scintillation counting (Beckman LS6500) (Hastie et al., 2006).

2.6. GST pulldown assay

GST-tagged MST2 and its SARAH domain protein coupled on glutathione-Sepharose beads were mixed with RAPL and its SARAH domain respectively at 4 °C for 1 h in 20 mM Hepes pH7.5, 100 mM NaCl, 1 mM DTT, and washed three times. The input and output samples were boiled and loaded on 12% SDS–PAGE followed by Coomassie blue staining.

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