Cellular Signalling 28 (2016) 488–497

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

The characterisation of LATS2 kinase regulation in Hippo-YAP signalling

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ARTICLE INFO

Article history:
Received 17 November 2015
Received in revised form 10 February 2016
Accepted 16 February 2016
Available online 18 February 2016

Keywords:
Intracellular kinase signalling
Hippo-YAP tumour suppressor pathway
Large tumour suppressor kinase
MOB proteins
STK3/4

ABSTRACT

By controlling the YAP1 proto-oncoprotein Hippo signalling plays important roles in cancer-associated processes. Current evidence suggests that the Hippo kinases MST1/2 together with the MOB1 scaffold protein promote the formation of active MOB1/LATS complexes which phosphorylate and thereby inhibit YAP1. However, the regulatory mechanisms of MST1/2-MOB1-LATS signalling are currently underinvestigated. Therefore, we studied LATS2 variants carrying specific modifications that mimic gain or loss of phosphorylation and/or abolish MOB1/LATS2 interactions. We discovered that Ser872 T-loop and Thr1041 hydrophobic motif (HM) phosphorylation of LATS2 is essential for LATS2 activation. MST1/2 phosphorylate LATS2 on Thr1041, but not Ser872, while MOB1 binding to LATS2 supports both phosphorylation events. Significantly, LATS2-PIF, a LATS2 variant containing the PRK2 HM, acts as a hyperactive LATS2 kinase that efficiently phosphorylates YAP1 and inhibits the transcriptional co-activity of YAP1. This inhibitory function of LATS2-PIF is dependent on LATS2 kinase activity, while MOB1/LATS2 and YAP1/LATS2 complex formation is dispensable, suggesting that elevated LATS2 kinase activity can be sufficient to oppose YAP1. Taken together, our characterisation of LATS2 variants uncovers novel insights into the regulation of LATS kinases in Hippo signalling.

1. Introduction

The Hippo pathway is important for the regulation of different cell biological processes [1–5], in particular cancer-associated cellular functions [6–8]. In mammalian Hippo core signalling, kinases are responsible for the tight regulation of the YAP1 proto-oncoprotein, whose deregulation is a hallmark of several human cancers [6–8]. The core is composed of the Hippo kinases MST1 and MST2 (also known as STK4 and STK3), the scaffold co-activator MOB1, the protein kinases LATS1 and LATS2, and the transcriptional co-activator YAP1 [9,10]. Current evidence suggests that MST1/2 and MOB1 function upstream of LATS1/2, and activated LATS1/2 subsequently inhibit YAP1 by phosphorylation [11]. However, the molecular regulation of LATS kinases by MST1/2 and MOB1 signalling is currently poorly understood.

LATS kinases are members of the LATS/NDR kinase family, a subgroup of AGC (protein kinase A (PKA)/PKG/PKC-like) kinases [10,12,13]. The human LATS/NDR family consists of four related serine/threonine protein kinases (LATS1, LATS2, NDR1/STK38, and NDR2/STK38L). Extensive biochemical studies have shown that, like many other AGC kinases, NDR1/2 kinases must be phosphorylated on their T-loop and hydrophobic motif (HM) for full activation [14–20]. However, LATS/NDR kinases are unique AGC kinases, being regulated through two conserved domains: a N-terminal regulatory region (NTR) juxtaposed to the catalytic domain, and a central insert in the catalytic domain right in front of the T-loop phosphorylation site [12]. MOB1 binding to the NTR plays a role in stimulating the T-loop and HM phosphorylation of NDR1/2 [14,15,21,22], where T-loop phosphorylation occurs *via* autophosphorylation, while HM phosphorylation is performed by members of the MST kinase family [15,18,22–24]. In spite of the rapid research progress in understanding mammalian Hippo signalling [1–8], these mechanistic insights into the regulation of NDR1/2 kinases by phosphorylation and MOB1 interactions must so far serve as the model for the regulation of LATS1/2 kinase [10].

It has been reported that MST1/2 can support the phosphorylation of LATS1/2 on Ser909/872 (T-loop sites) and Thr1079/1041 (HM sites) [25] and that T-loop and HM phosphorylation of LATS1 is essential for LATS1 kinase activation [26]. However, it is currently unknown whether MST1/2 phosphorylates these sites directly and whether LATS2 is regulated through T-loop and HM phosphorylations. Furthermore, although MOB1/LATS1 and MOB1/LATS2 complexes have been described [10,21,26–28] and LATS1 mutants defective in MOB1 binding were studied [26,29], the regulation of LATS1/2 phosphorylation through MOB1 binding is poorly understood, although the phosphorylation status of S909/

Abbreviations: LATS2, large tumour suppressor 2; MST1/2, mammalian serine/threonine Ste20-like kinases 1/2; MOB, Mps one binder; NDR, nuclear Dbf2-related; NTR, N-terminal regulatory region; HM, hydrophobic motif; PIF, PDK1-interacting fragment; PRK2, PKC related kinase 2; STK, serine/threonine kinase; YAP1, yes-associated protein 1; wt, wild-type; kd, kinase-dead.

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http://dx.doi.org/10.1016/j.cellsig.2016.02.012
0898-6568/

S872 and T1079/T1041 of LATS1/2 kinases is frequently used to check the activity status of mammalian LATS1/2 kinases (for examples see Refs. [29–32]). Collectively, these findings (or the lack thereof) illustrate that studies dissecting the molecular regulation of LATS1/2 kinases by MST1/2 and MOB1 signalling are urgently needed.

Here, we focus on defining the regulation of human LATS2 kinase by MST1/2 and MOB1, since (a) LATS2, but not LATS1 deficiency, results in embryonic lethality [28,33,34], (b) LATS2 contains only one PPXY motif (in contrast to LATS1) [35], hence PPXY-mediated interactions are more easily manipulated in LATS2, and (c) LATS1 phospho-acceptor and MOB1 binding deficient mutants have already been described [26,27,29]. We designed and characterised LATS2 variants carrying point mutations altering phospho-acceptor residues and/or abolishing MOB1 binding. Collectively, our study is the first demonstration that the Hippo MST1/2 kinases specifically phosphorylate LATS2 on the HM at Thr1041, but not the activation T-loop at Ser872, while MOB1 binding to the NTR of LATS2 supports Ser872 and Thr1041 phosphorylation. LATS2-PIF, a LATS2 version containing the PRK2 HM, is constitutively hyperactive independent of MOB1/LATS2 complex formation. LATS2-PIF phosphorylates YAP1 and inhibits the transcriptional co-activity of YAP1 independent of MOB1/LATS2 and YAP1/LATS2 complex formation, suggesting that elevated LATS2 kinase activity can be sufficient to oppose YAP1 function.

2. Materials and methods

2.1. Cell culture, transfections, and cell treatments

Cos-7 and HEK293 cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS). Exponentially growing Cos-7 and HEK293 cells were plated at a consistent confluence and transfected with plasmids using Fugene 6 (Promega) or Metafectene Pro (Biontex) according to the manufacturer's instructions as described [15,36]. Treatments with okadaic acid (OA; Enzo Life Sciences) were performed as described [15].

2.2. Immunoblotting, immunoprecipitations, and antibodies

Immunoblotting and co-immunoprecipitation experiments were performed as described [15,16,22]. The characterisation of LATS2 binding to MOB1A, MOB1B, or YAP1 was carried out in low-stringency buffer as defined previously [15]. Rabbit polyclonal anti-Ser872-P and anti-Thr1041-P antibodies were raised against recombinant phosphopeptides coupled to KLH. Rabbit injections/bleed collections were done by Eurogentec. Anti-phospho-peptide antibodies were purified as described [16,37]. Anti-HA 12CA5, anti-myc 9E10, and anti-α-tubulin YL1/2, antibodies have been described [15,16,23,24]. Anti-GAPDH was from Millipore (Mab374). Additional anti-HA antibodies were from Cell Signaling (C29F4) and Roche (3F10). Anti-myc (71D10), anti-MST1 (3682), anti-MST2 (3952), anti-S127-P (13,008), anti-S397-P (13,619), and anti-YAP1 (4912) were from Cell Signaling. Anti-LATS2 (A300-479A) was from Bethyl laboratories. All secondary antibodies were purchased from GE Healthcare (NA931, NA934, and NA935).

2.3. HA-LATS2 kinase assays

The analyses of kinase activities of immunoprecipitated HA-LATS2 versions was performed as described [16,26]. [γ-P32]-ATP was obtained from Hartmann Analytic GmbH. The LATS substrate peptide (KKRNRRLSVA) was from Biomatik.

2.4. GST-MST1/2 kinase assays

A previously established kinase assay [38] using GST-MST1 as kinase and HA-NDR1 as substrate was adjusted as follows: to produce immunopurified full-length HA-tagged LATS2 kinase-dead, Cos-7 cells were transfected and processed for immunoprecipitation using anti-HA antibody as described earlier [16]. Immunopurified proteins were washed twice with MST1/2 kinase buffer (5 mM Tris pH 7.5, 2.5 mM beta-glycerophosphate, 1 mM EGTA, 1 mM Na_3VO_4, 4 mM $MgCl_2$, 0.1 mM DTT), before incubating at 30 °C for 30 min in 20 μl of reaction buffer (5 mM Tris pH 7.5, 100 μM ATP, 2.5 mM beta-glycerophosphate, 1 mM EGTA, 1 mM Na_3VO_4, 4 mM $MgCl_2$, 0.1 mM DTT, 10 μCi of [γ-^{32}P]ATP [3000 Ci/mmol; Hartmann Analytic]) in the absence or presence of GST-MST1/2 (100 ng per reaction). The reactions were stopped by the addition of Laemmli buffer, before the proteins were separated by SDS-PAGE, followed by immunoblotting. Recombinant GST-MST1 (M9697) and GST-MST2 (S6573) were from Sigma.

2.5. Immunofluorescence microscopy

Cells were processed for immunofluorescence as defined [16,39]. Briefly, cells were fixed in ice-cold methanol for 5 min at −20 °C, rehydrated in PBS and incubated with anti-HA (C29F4; Cell Signaling) antibody. Alternatively, cells were fixed (3% paraformaldehyde, 2% sucrose in PBS pH 7.4) for 20 min at room temperature, before permeabilization using Triton X-100 in PBS. The anti-rabbit FITC antibody was from Jackson ImmunoResearch. DNA was counterstained with TO-PRO-3 iodide (Invitrogen). Coverslips were mounted in Vectashield medium (Vector Lab.). Images were obtained with a LSM510 meta confocal laser scanning microscope (Carl Zeiss Ltd.). Images were processed using Imaris 4.0 (Bitplane AG) and Photoshop CS5 (Adobe Systems Inc.).

2.6. Luciferase reporter assays

The transcriptional co-activity of YAP1 was assessed as described [40]. HEK293 cells were transfected with the indicated plasmid combinations using jetPEI (Polyplus transfection) according to the manufacturer's instructions. 48 h post-transfection cells were processed to measure luciferase activities using the Dual-Luciferase Reporter Assay System (E1910) as defined by the manufacturer (Promega). Constitutive expression of Renilla luciferase (pRL-CMV Renilla luciferase) served as transfection control, while Firefly luciferase expression (pGL3b 8xGTIIC TEAD luciferase reporter, Addgene plasmid #34615) was used to monitor the transcriptional co-activity of YAP1 as reported [40].

2.7. Construction of plasmids

hMOB1A, hMOB1B, and YAP1 cDNAs cloned in pcDNA3-based vectors were described previously [15,16,22,39,41]. pRL-CMV Renilla luciferase and pGL3b 8xGTIIC TEAD luciferase reporter plasmids were kindly provided by Pablo Rodriguez-Viciana (UCL Cancer Institute) and the Piccolo laboratory (Addgene plasmid #34615). LATS2 wild-type (wt) was amplified by PCR and subcloned into pcDNA3_HA using *Bam*HI and *Xho*I. pcDNA3_HA-LATS2(wt) served as template for the generation of the following LATS2 mutants by PCR-based mutagenesis: Y518A, Y518F, K620A, R623A, S653A, R654A, R657A, R660A, D809A (kinase-dead, kd), S872A, T1041A, T1041D, T1041E, PIF, PIF/kd, S872A/PIF, R623A/PIF, R657A/PIF, R623A/PIF/kd, R657A/PIF/kd, Y518A/PIF, Y518/R623A/PIF, and Y518A/R657A/PIF. All constructs were confirmed by sequence analysis of the entire cDNAs. Further details on the generation of plasmids and sequences of primers are available upon request.

2.8. Statistical analysis

Graphics and statistical analyses were carried out using the GraphPad Prism software. Data are presented as mean ± s.e.m., unless stated otherwise. The significance of differences between the means or the population distributions was determined using two-tailed unpaired Student *t*-test. For all tests, differences were considered statistically significant when