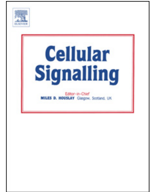
Cellular Signalling 26 (2014) 1657–1667

Contents lists available at ScienceDirect

Cellular Signalling

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



Constitutively active NDR1-PIF kinase functions independent of MST1 and hMOB1 signalling

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ARTICLE INFO

Article history:
Received 18 March 2014
Accepted 8 April 2014
Available online 18 April 2014

Keywords:
Intracellular kinase signalling
MST1/STK4
NDR1/STK38
MOB proteins
Hydrophobic motif phosphorylation
Centrosome duplication

ABSTRACT

The human MST1/hMOB1/NDR1 tumour suppressor cascade regulates important cellular processes, such as centrosome duplication. hMOB1/NDR1 complex formation appears to be essential for NDR1 activation by autophosphorylation on Ser281 and hydrophobic motif (HM) phosphorylation at Thr444 by MST1. To dissect these mechanistic relationships in MST1/hMOB1/NDR signalling, we designed NDR1 variants carrying modifications that mimic HM phosphorylation and/or abolish hMOB1/NDR1 interactions. Significantly, the analyses of these variants revealed that NDR1-PIF, an NDR1 variant containing the PRK2 hydrophobic motif, remains hyperactive independent of hMOB1/NDR1-PIF complex formation. In contrast, as reported for the T444A phospho-acceptor mutant, NDR1 versions carrying single phospho-mimicking mutations at the HM phosphorylation site, namely T444D or T444E, do not display increased kinase activities. Collectively, these observations suggest that in cells Thr444 phosphorylation by MST1 depends on the hMOB1/NDR1 association, while Ser281 autophosphorylation of NDR1 can occur independently. By testing centrosome-targeted NDR1 variants in NDR1- or MST1-depleted cells, we further observed that centrosome-enriched NDR1-PIF requires neither hMOB1 binding nor MST1 signalling to function in centrosome overduplication. Taken together, our biochemical and cell biological characterisation of NDR1 versions provides novel unexpected insights into the regulatory mechanisms of NDR1 and NDR1's role in centrosome duplication.

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1. Introduction

Most signal transduction cascades transmit signals through protein kinases, which consequently represent one of the largest superfamilies found in the human genome [1]. In particular members of the AGC (protein kinase A (PKA)/PKG/PKC-like) subfamily of protein kinases have crucial cellular functions in cell growth, metabolism, proliferation and survival [2]. All AGC kinases share structural similarities, and many AGC kinases require phosphorylation of two conserved regulatory sites for activation: one conserved Ser/Thr residue within the activation segment (also termed T-loop) and one within the C-terminal hydrophobic motif (HM). As exemplified by the biochemical and structural characterization of the AGC kinase, Akt/PKB [3,4], both regulatory sites must be phosphorylated simultaneously to achieve full kinase activation.

The NDR (nuclear Dbf2-related)/LATS (large tumour suppressor) kinase family is a subgroup of AGC kinases and consists of four related serine/threonine protein kinases (NDR1/STK38, NDR2/STK38L, LATS1, and LATS2) in the human genome [1,5]. In mammals, LATS1/2 kinases are central to the Hippo tumour suppressor pathway [6–9], and NDR kinases regulate essential processes, such as centrosome duplication [10,11], cell cycle/mitotic progression [12–15], ciliogenesis [16], neuronal dendrite/synapse formation [17], and apoptotic signalling [18,19], where the latter function appears to be important for suppression of tumour growth [20]. In spite of the rapid progress in deciphering functions of mammalian LATS1/2, the mechanism of NDR regulation by phosphorylation must serve as a model for LATS regulation [8]. Therefore, studies addressing the regulatory mechanisms of NDR kinases are also very relevant for understanding the regulation of mammalian LATS-Hippo signalling.

Previously, it was reported that human NDR1 is dramatically activated upon inhibition of protein phosphatase 2A by okadaic acid (OA) [21,22], which is not surprising since NDR1, like many other AGC kinases, must be phosphorylated on Ser281 (T-loop phosphorylation) and Thr444 (HM phosphorylation) for full activation [21–25]. However, NDR kinases are unique among AGC kinases because they have a distinctive activation mechanism. Competitive binding of the co-activator hMOB1 or the inhibitor hMOB2 to a conserved N-terminal regulatory (NTR) domain of NDR1 regulates kinase activity [23,24,26]. Initially, it was thought that binding of hMOB1 to the NTR only stimulates Ser281 autophosphorylation, but recent evidence suggests that hMOB1/NDR complex formation plays also a role in HM phosphorylation of NDR1,

Abbreviations: NDR1, nuclear Dbf2-related 1; MST1, mammalian serine/threonine Ste20-like kinase 1; STK, serine/threonine kinase; MOB, Mps one binder; HM, hydrophobic motif; PRK2, PKC related kinase 2; LATS, large tumour suppressor; PIF, PDK1-interacting fragment.

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http://dx.doi.org/10.1016/j.cellsig.2014.04.011
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since Thr444 phosphorylation by MST1 is hMOB1 dependent in human cells [10,27]. Therefore, NDR1 is regulated by a multistep process involving HM phosphorylation on Thr444, autophosphorylation of Ser281, and NTR binding of hMOB1. However, the order and dependency of these regulatory events are currently unknown [8].

Here, we dissected these three signalling steps by examining the basal and OA-stimulated activities of NDR1 variants carrying modifications that mimic HM phosphorylation and/or abolish hMOB1/NDR1 complex formation. These efforts revealed that NDR1-PIF, an NDR1 variant containing the PRK2 hydrophobic motif, is hyperactive and maintains high levels of Ser281 autophosphorylation despite loss of hMOB1 binding. Moreover, by studying NDR1-PIF in context of the previously reported MST1/hMOB1/NDR1 centrosome duplication signalling cascade [10], we demonstrate that expression of centrosome targeted and hMOB1-binding deficient NDR1-PIF is sufficient to restore centrosome amplification in MST1-depleted cells, thereby showing that NDR1-PIF can function independent of hMOB1 and MST1 signalling in human cells.

2. Materials and methods

2.1. Cell culture and transfections

Cos-7, U2-OS and PT67 cells were maintained in DMEM supplemented with 10% foetal calf serum. Exponentially growing Cos-7 cells were plated at a consistent confluence (1×10^6 cells/10-cm dish) and transfected the next day using Fugene 6 (Roche) or Metafectene Pro (Biontex) as described by the manufacturer. U2-OS cells were transfected with Lipofectamine 2000 (Invitrogen) as described by the manufacturer.

2.2. Cell treatments

In OA experiments, cells were treated for 60 min with 1 μM okadaic acid (OA; Enzo Life Sciences). Aphidicolin and tetracycline were purchased from Sigma.

2.3. Generation of stable cell lines

U2-OS Tet-on cells expressing tetracycline-regulated shRNA against NDR1, hMOB1A/B, or MST1 have been described previously [10,11]. Retroviral pools of rescue cell lines were generated and maintained as described elsewhere [11].

2.4. Construction of plasmids

Human NDR1, hMOB1A, hMOB1B, and hMOB2 pcDNA3-based vectors have been described previously [10,11,24]. New NDR1 mutants described herein were generated by PCR-based mutagenesis. NDR1 cDNAs refractory to siRNA were generated by PCR mutagenesis introducing silent mutations into residues 128 to 134 of human NDR1 (termed 6N for changing 6 nucleotides in the siRNA targeting sequence) [11]. To obtain centrosome-targeted NDR1 constructs, human NDR1 cDNAs were subcloned into a pcDNA3-HA (hemagglutinin)-AKAP derivative using *Bam*HI and *Xho*I [11]. For rescue experiments NDR1 cDNAs and their corresponding N-terminal tags were subcloned into the retroviral vector pCMV (neo-retro) [28]. All constructs were confirmed by sequence analysis. Further details of the generation of constructs are available upon request.

2.5. Antibodies

The generation and purification of anti-Ser281-P, anti-NDR_{CTD}, and anti-hMOB1A/B antibodies have been described previously [10,11,22,24]. Mouse monoclonal antibodies against human STK38/NDR1 were purchased from AbD Serotec and Abnova, respectively. Rat monoclonal anti-α-tubulin (YL1/2) and mouse monoclonal anti-HA 12CA5 and anti-myc 9E10 antibodies were used as hybridoma supernatants. Additional anti-HA antibodies were from Cell Signaling (C29F4) and Roche (3F10). Anti-myc (71D10) and anti-MST1 were from Cell Signaling. Anti-γ-tubulin (GTU-88) was from Sigma. Secondary antibodies were purchased from GE Healthcare.

2.6. Immunoblotting and immunoprecipitation

Immunoblotting and co-immunoprecipitations (co-IPs) were performed as described [24]. The characterisation of NDR1 binding to hMOB1/2 was performed in low-stringency buffer (30 mM HEPES pH 7.4, 20 mM beta-glycerophosphate, 20 mM KCl, 1 mM EGTA, 2 mM NaF, 1 mM Na3VO4, 1% TX-100) supplemented with protease inhibitors.

2.7. HA-NDR kinase assays

Analysis of immunoprecipitated HA-NDR species was performed as described previously [24]. [γ-P^{32}]-ATP was from Hartmann Analytic GmbH. The NDR substrate peptide (KKRNRRLSVA) was purchased from Biomatik.

2.8. Immunofluorescence microscopy

Cells were processed for immunofluorescence as defined elsewhere [11,24]. In brief, cells were fixed in ice-cold methanol for 5 min at −20 °C, then rehydrated in PBS for 5 min at room temperature and incubated with anti-γ-tubulin (GTU-88; Sigma) and anti-HA (C29F4; Cell Signaling) antibodies. Secondary antibodies were from Jackson ImmunoResearch. DNA was counterstained with TO-PRO-3 iodide (Invitrogen) or DAPI (Sigma). Coverslips were mounted in Vectashield medium (Vector Lab.). Images were obtained with a LSM510 meta confocal laser scanning microscope (Carl Zeiss Ltd.). Photographic images were processed using Imaris 4.0 (Bitplane AG) and Photoshop CS4 (Adobe Systems Inc.).

3. Results

3.1. PIFtide based hydrophobic motif (HM) modifications in human NDR1 result in hyperactive NDR1 kinases, while phospho-mimetic T444D and T444E mutations do not

To develop a research tool allowing the examination of the importance of HM phosphorylation of human NDR1, we characterised NDR1 variants carrying different HM modifications (Figs. 1 and 2). Like the majority of AGC kinases, NDR1 is stimulated by C-terminal HM phosphorylation on Thr444 phosphorylation [2,5]. To test whether Asp/Glu substitutions at Thr444 can mimic Thr444 phosphorylation, thereby possibly generating constitutively active NDR1, we generated NDR1 mutants carrying Thr444 to Asp (T444D) or Glu (T444E) substitutions (Fig. 1A). Next, immunoprecipitated NDR1 wild-type (wt), kinase-dead (kd; K118A), (T444D) and (T444E) were subjected to kinase assays (Fig. 1B and C, lanes 1 to 4). Confirming the specificity of our peptide kinase assay [24], NDR1(kd) did not display any activity (Fig. 1C). NDR1(T444D) and NDR1(T444E) did not show increased activities when compared to NDR1(wt) (Fig. 1C). Given that these Asp/Glu modifications are known to elevate the basal kinase activities of other AGC kinases (for example Ser473D/Ser474D in Akt/PKB kinases dramatically increases basal Akt/PKB activities [3,29]), this observation suggests that single phospho-mimetic mutations at the HM of NDR1, namely T444D or T444E, are not sufficient to trigger NDR1 activation.

As an alternative approach to mimic HM phosphorylation of human NDR1, we introduced additional changes to the HM of NDR1. Based on the observation that the HM of PRK2 (PKC related kinase 2) functions as a potent mimic of HM phosphorylation in some AGC kinases, such as Akt/PKB [3,4], the C-terminus of NDR1 was replaced by the HM of

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