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AMOTL2 interaction with TAZ causes the inhibition of surfactant proteins expression in lung cells

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ABSTRACT

Background: TAZ (Transcriptional co-Activator with PDZ-binding motif), is a biologically potent transcriptional coactivator and functions by binding to the PPXY motif present in several transcription factors. Notably, TAZ behaves as a transducer linking cytoplasmic signaling events to transcriptional regulation in the nucleus. Several different factors regulate TAZ expression and/or function. In particular, a major regulation of TAZ activity occurs through the Hippo pathway by a phosphorylation-mediated mechanism that causes its cytoplasmic sequestration or degradation.

Results: Here we demonstrate that AMOTL2 robustly co-immunoprecipitates with TAZ, and their interaction is dependent on the WW domain of TAZ and the PPXY motif in the N-terminus of AMOTL2. Furthermore, we show that AMOTL2 colocalizes with TAZ in the cytoplasm of H441 human lung cells and regulates TAZ cytoplasm-to-nucleus translocation through direct protein–protein interaction. Interestingly, the overexpression of AMOTL2 inhibits the functional cooperation between the transcription factor TTF-1 and TAZ on the Surfactant C gene promoter, as well as the expression of other known target genes of these regulatory factors.

Conclusions: Taken together, our results suggest an inhibitory role of AMOTL2 on TAZ ability to co-activate transcription and describe a different mechanism, Hippo pathway-independent, that modulates the activity of TAZ in lung cells through the interaction with Angiomotin-like 2 (AMOTL2).

1. Introduction

TAZ (also referred as Wwtr1) was initially discovered as a 14-3-3-binding molecule (Kanai et al., 2000). Most human and mouse tissues, except thymus and peripheral blood leukocytes, express TAZ mRNA but the highest levels of expression were observed in kidney, heart, and lung. Sharing amino acid sequence homology with YAP (Yes-Associated Protein), TAZ contains a 14-3-3 binding site, a central WW domain and a PDZ-binding motif at the COOH terminus that localizes TAZ into discrete nuclear foci. TAZ functions as a transcriptional co-activator through the binding of its WW domain to the (L/P)PXY amino acid motif present on several transcription factors. The Hippo pathway represents a major regulatory control to restrict the activity of TAZ and it does so by affecting its subcellular distribution and stability. Upon activation, the protein kinase complex LATS1/2-Mob1 phosphorylates multiple Ser residues within the HXRXXS motif of TAZ (Lei et al., 2008). In particular, phosphorylation of the serine residue at position 89 is responsible for its interaction with 14-3-3 proteins in the cytoplasm. Differently when dephosphorylated, TAZ is translocated in the nucleus where it functions as a transcriptional coactivator (Hong et al., 2005) for several transcription factors, including TTF-1 (Park et al., 2004), Cbfa1/Runx2 (Cui et al., 2003), polyomavirus T antigens (Tian et al., 2004), TEF-1 (Mahoney et al., 2005), TBX5 (Murakami et al., 2005), Pax3 (Murakami et al., 2006) and Pax8 (Di Palma et al., 2009). Consequently, TAZ is implicated in several biological processes such as mesenchymal stem cell differentiation (Hong et al., 2005), embryogenesis (Murakami et al., 2006), cell proliferation and promotion of epithelial–mesenchymal transition (Lei et al., 2008). TAZ also plays a role in cancer, in fact it has been reported overexpressed in a significant fraction of primary breast cancers (Chan et al., 2008), in papillary thyroid carcinoma (de Cristofaro et al., 2011) and importantly involved in the tumorigenicity of NSCLC cells (Zhou et al., 2011). Moreover, it has been recently published that the carboxy-terminal PDZ binding motif of TAZ interacts with the first PDZ domain of zona occludens-1 (ZO-1) and 2 (ZO-2) proteins, suggesting that selected junction-associated proteins might control TAZ nuclear translocation and activity (Remue et al., 2010). Studies from three independent laboratories have shown that

Abbreviations: TAZ, Transcriptional co-Activator with PDZ-binding motif; AMOTL2, Angiomotin-like 2 protein; TTF-1, Thyroid Transcription Factor-1; YAP, Yes-Associated Protein; GST, glutathione-S transferase; MLE, mouse lung epithelial; HA, hemagglutinin; CMV, cytomegalovirus; cDNA, DNA complementary to RNA; PBS, phosphate buffered saline; SP-A, SP-B, SP-C, surfactant protein A, B, C; PCR, polymerase chain reaction; BMP4, bone morphogenetic protein 4.

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the inactivation of the mouse *Taz* gene results in the formation of multiple cysts in the kidney characteristic of polycystic kidney disease (PKD) and emphysema-like features including enlarged air space and low elastance in the lung (Hossain et al., 2007; Makita et al., 2008; Tian et al., 2007). In this study, we report the interaction between TAZ and the Angiomotin-like 2 protein in human lung cells and we demonstrate that this tight junction-associated protein is able to modulate the transcriptional properties of TAZ.

2. Materials and methods

2.1. Plasmids

The plasmids used have been previously described and are as follows: GST-TAZ, CMV-TAZ and 4.8 mSP-C-luc (Park et al., 2004), CMV-TTF-1 (Bohinski et al., 1994), GST-NW-TAZ, GST-W-TAZ and GST-C-TAZ (Di Palma et al., 2009). The FLAG-TAZ was generated by PCR amplification of mouse TAZ cDNA and subcloning in the NotI-XbaI sites of 3xFLAG-CMV10 (Sigma). pCAGGS-HA-AMOTL2 full length and pCAGGS-HA-AMOTL2-(ΔPDZ) were kindly provided by S. Sukita and MYC-AMOTL2-(Y213A) was kindly provided by J. Chen. MYC-AMOTL2 was generated by PCR amplification of human AMOTL2 cDNA and subsequent subcloning in the EcoRI-XbaI sites of the pcDNA 3.1 vector (Invitrogen).

2.2. In-gel digestion, mass spectrometry analysis and protein identification

GST and GST-TAZ proteins were loaded onto glutathione-Sepharose beads (GE Healthcare, Waukesha, WI, USA) at a concentration of 1 μg/μl packed beads for 20 min at 4 °C. A total of 5×10^8 H441 cells were lysed; after a pre-clearing step on GST-coated beads, the total extract was challenged with GST and GST-TAZ loaded beads. After washing, the bound proteins were separated by SDS–PAGE on an 8–15% gradient gel and stained with NOVEX colloidal blue staining kit (Invitrogen); the gel image was then acquired by using an Image Scanner III densitometer (GE Healthcare Life Sciences). Gel lanes corresponding to the bait and mock samples were manually cut into 18 slices, which then were triturated and washed with water. Proteins were *in-gel* reduced, S-alkylated and digested with trypsin as previously reported (D'Ambrosio et al., 2008). Digest aliquots were removed and subjected to a desalting/concentration step on a ZipTip mC_{18} disk (Millipore) using 5% formic acid/50% acetonitrile as eluent before nanoLC-ESI-LIT-MS/MS analysis.

Digests were analyzed by nanoLC-ESI-LIT-MS/MS using a LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Odense, Denmark). Peptide mixtures were separated on an Easy C_{18} column (100 × 0.075 mm, 3 mm) (Proxeon, Odense, Denmark) by using a linear gradient of acetonitrile containing 0.1% formic acid in aqueous 0.1% formic acid; acetonitrile was ramped from 5% to 40% over 60 min, and from 40% to 80% in 10 min, at a flow rate of 300 nl/min. Spectra were acquired in the range *m/z* 400–2000. A duplicate analysis of each sample was realized by using an acquisition method performing either (i) a data-dependent product ion scanning procedure over the three most abundant ions, enabling dynamic exclusion (repeat count 1 and exclusion duration 1 min), or (ii) as the previous method, with exclusion of the peptides confidently identified in the first run, in order to increase both the number of the identified proteins and the corresponding sequence coverage. In both cases, the mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

A Proteome Discoverer platform (ThermoFisher, San Jose, CA, USA) was utilized to search raw mass data against an updated Uniprot non-redundant sequence database (2010/4), using both Sequest (ThermoFisher, USA) and Mascot (Matrix Science, UK) algorithms. Database searching was performed by using a mass tolerance value of 2.2 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, a missed cleavages maximum value of 2 and Cys carbamidomethylation and Met oxidation as fixed and variable modification, respectively. Candidates with more than 2 assigned peptides with an individual Sequest X_{Corr} value depending on charge state (>1.5 for charge state 1, >2.0 for charge state 2, >2.5 for charge state 3, >3.0 for charge state 4), with probability >2 and filtered by MASCOT score >20, with a significant threshold $p < 0.05$, were further considered for protein identification. Finally, TAZ-interacting proteins were identified by subtracting the components ascertained for the bait with that ones from the corresponding control.

2.3. Cell culture, transfections and reporter assays

H441 cells (kindly provided by J. Whitsett) were grown in RPMI medium (Euroclone) supplemented with 10% fetal bovine serum. For stable transfection of pCAGGS-HA-AMOTL2, H441 cells were cultured in 100 mm plates and transfected with 2 μg of DNA with the addition of 200 ng of pBABE-puro. 48 h after transfection, selection with the specific antibiotic (0.4 μg/ml puromycin, Sigma) was started. MLE-15 cells were cultured as described (Wikenheiser et al., 1993). For stable transfection of MYC-AMOTL2 and MYC-AMOTL2-(Y213A), MLE-15 cells were cultured in 100 mm plates and transfected with 2 μg of DNA; 48 h after transfection, selection with the specific antibiotic (400 ng/ml geneticin, GIBCO-Life Technologies) was started. HeLa cells were grown in Dulbecco's modified Eagle's medium (Euroclone) supplemented with 10% fetal bovine serum (Hyclone). For transient transfection experiments, cells were plated at 8×10^4 cells/12-well tissue culture dish 5 to 8 h prior to transfection. Transfections were carried out with the FuGENE6 reagent (Promega) according to the manufacturer's directions. The DNA/FuGENE ratio was 1:3 in all the experiments. Firefly luciferase activity was normalized on the activity of the pRL-Renilla vector in order to correct each sample for transfection efficiency. The total amount of transfected DNA was kept constant with an empty expression vector in all the transfection assays.

Luciferase and Renilla assays were performed using the Luciferase Assay System and the Renilla Luciferase Assay System following the manufacturer's directions (Promega). Luminescence was measured with the LUMAT LB 9507 luminometer (Berthold Technologies). Transfection experiments were done in duplicate and repeated at least three times. Statistical analysis has been performed by means of an unpaired two-tailed Student's t test to obtain the P value associated with the observed fold of activation differences.

2.4. Protein extracts and immunoblotting

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in JS buffer containing 50 mM Hepes pH 7.5, 150 mM NaCl, 5 mM EGTA pH 7.8, 10% glycerol, 1% Triton, 1.5 mM MgCl2, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF). Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA) and Western blots were performed as described (Di Palma et al., 2009). The primary antibodies used were: anti-TAZ (kindly provided by M. Sudol), anti-AMOTL2 (kindly provided by S. Tsukita), anti-Myc (Santa Cruz, CA), anti-HA (Covance), anti-FLAG (Sigma), and anti-GST (kindly provided by P.P. Di Fiore). Horseradish peroxidase was detected with ECL (Pierce).

2.5. Pull down assay and co-immunoprecipitation

GST-TAZ and deletion mutant proteins were generated as previously described (Park et al., 2004). Pull-down assays were performed by challenging 4 μg of GST or GST-TAZ, GST-NWTAZ, GST-WTAZ or GST-CTAZ purified proteins, previously bound to glutathione-agarose beads, with protein extracts from H441 or HeLa cells transiently transfected with HA-AMOTL2, HA-AMOTL2-(ΔPDZ) or MYC-AMOTL2-(Y213A). The binding reactions were carried out for 90 min at 4 °C on a rotating wheel and then the beads were washed several times with the JS buffer.