TAZ interacts with zonula occludens-1 and -2 proteins in a PDZ-1 dependent manner

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ABSTRACT

The transcriptional coactivator TAZ recognizes L/PPxY motifs in transcription factors like Runx1/2 through its WW domain. We show that the first PDZ domain of zona occludens-1 (ZO-1) and 2 (ZO-2) interacts with the carboxy-terminal PDZ binding motif of TAZ. Deletion of this motif abrogates binding. ZO-2 colocalizes with TAZ in the nucleus of MDCK cells and ZO-2 expression alters TAZ localization in human embryonic kidney cells. Luciferase assays demonstrate ZO-2 inhibition of TAZ-mediated transactivation. We propose that zonula occludens is a negative regulator of TAZ and suggest that selected tight junction proteins control nuclear translocation and activity of TAZ.

1. Introduction

TAZ (transcriptional coactivator with PDZ binding motif), also known as WWTR1 (WW domain containing transcription regulator 1) was first identified as a 14-3-3 binding protein and is a paralog of YAP (Yes-associated protein) [1]. Both TAZ and YAP share a strongly conserved carboxy-terminal postsynaptic density95-discs large-zonula occludens (PDZ) binding motif (-FLTWL). Since they lack a DNA binding domain, stimulation of gene expression is...
dependent on the interaction of the WW domain with PProX motifs in transcription factors. TAZ has been reported to bind Runx2 [2], PPARγ [3], and the TEF-1/TEAD family [4]. By consequence, TAZ is implicated in several biological processes such as mesenchymal stem cell differentiation [3], embryogenesis [5], cell proliferation and promotion of epithelial-mesenchymal transition [6]. TAZ also plays a role in cancer [7].

The mechanisms that control functions of YAP and TAZ are starting to be elucidated. Both proteins are part of the Hippo pathway originally identified in Drosophila [8]. All known components of this kinase cascade are highly conserved in vertebrates where they are also responsible for controlling tissue and organ size by coordinating proliferation and apoptosis. TAZ activity is also regulated by its C-terminal PDZ binding motif that is indispensable for TAZ-stimulated gene transcription and accumulation into nuclear foci [1]. Currently, the only known PDZ domain containing interaction partner for TAZ is NHERF-2 (Na+/H+ exchanger regulatory factor), a polypeptide that tethers plasma membrane ion channels and receptors to cytoskeletal actin. TAZ specifically associates to the first PDZ domain of NHERF-2, whereas YAP interacts with the second PDZ domain [9].

Although the importance of the PDZ binding motif of TAZ is clearly acknowledged, the PDZ protein responsible for regulation of TAZ localization and function is not known. Several junction-associated proteins are not solely confined at the plasma membrane but belong to a growing class of nucleo-junctional proteins that shuttle between the junction and the nucleus [10]. This is illustrated by the interaction of ZO-2 with the transcription factors Fox, Jun and CCAAT/enhancer binding protein [11]. These tight junction proteins are promising candidates to transmit signals from the membrane and cytoskeleton to the nucleus, thereby potentially regulating TAZ-mediated transcription. Here we report the interaction between TAZ and Zonula Occludens-1 and -2 (ZO-1/-2). These ligands also interact with YAP [12].

2. Materials and methods

2.1. Antibodies

Custom made rabbit polyclonal antibodies for human TAZ were generated (Supplementary Fig. 1). Rabbit anti-green fluorescent protein (GFP) was from Santa Cruz (Santa Cruz, California, USA). Rabbit anti-ZO-1, rabbit anti-ZO-2, mouse anti-ZO-1 and mouse anti-ZO-2 were from Invitrogen (Merelbeke, Belgium), mouse anti-Myc from Millipore (Billerica, Massachusetts, USA), rat anti-α-HA was from Roche and rabbit anti-glutathione-S-transferase (GST) from Abcam. All secondary antibodies used for immunofluorescence were obtained from Molecular Probes (Merelbeke, Belgium).

2.2. Plasmids

Supplementary data.

2.3. Protein expression

GST–PDZ fusions were transformed into BL21(DE3) pLysS cells. Cells were grown at 37°C in LB medium containing 50 μg/ml ampicillin. Protein expression was induced at 37°C for 4 h with IPTG. The PDZ domains were purified by glutathione sepharose affinity chromatography according to instructions of the manufacturer.

2.4. GST pull down, immunoprecipitation, and immunoblotting

HEK293T cells were transfected with eukaryotic expression vectors for GST, GST–TAZ wild-type or GST–TAZ APDZ, or co-transfected with GFP–ZO-1 or GFP–ZO-2. Cells were disrupted in ice-cold lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1% Triton-X100, 1 mM PMSF) and a protease inhibitor cocktail mix (Roche Diagnostics, Mannheim, Germany). Insoluble material was removed by centrifugation (20 000 × g for 15 min at 4°C). Cleared extracts were incubated with glutathione sepharose (GE Healthcare). Proteins were separated by SDS–PAGE and transferred to nitrocellulose. For GST pull down assays, 10 μg of the GST fusion proteins bound to glutathione sepharose beads were incubated at 4°C for 1 h with 1 μg cytoplasmic protein. Interacting proteins were processed as described above.

2.5. Western blotting

Protein concentrations were determined by the method of Bradford [13] using bovine serum albumin as a standard. Western

![Fig. 1](image-url) Interaction between ZO proteins and TAZ is established through recognition of the C-terminal PDZ binding motif. (A) HEK293T cells were co-transfected with GST, GST–TAZ wild-type (WT), GST–TAZ without the PDZ binding motif (TAZ APDZ) and GFP–ZO-1 or GFP–ZO-2. Cells extracts were incubated with glutathione sepharose. Total lysates (top panels, input) show expression of transfected constructs. Immunoblotting with a polyclonal anti-GFP antibody reveals ZO-1 and ZO-2 binding to wild-type GST TAZ (middle panels, pull down) in contrast to mutant TAZ lacking the C-terminal PDZ binding motif. Sizes in kDa are indicated. Bottom panels show Coomassie stained gels with asterisks highlighting ZO-1 and ZO-2. (B) GST pull down from HEK cells transfected with GST, GST–TAZ WT and GST–TAZ APDZ. Endogenous ZO proteins were detected with polyclonal anti-ZO-1 (left panel) and ZO-2 antibodies (right panel) showing interaction with the WT GST TAZ but not with GST TAZ APDZ. The input of endogenous ZO proteins is shown in the upper panel.
blotting was performed as described [14]. Proteins were visualized by enhanced chemiluminescence detection (ECL, GE Healthcare).

2.6. Cell culture

Cells were maintained in Dulbecco’s modified Eagle’s medium + Glutamax (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml penicillin and 0.1 mg/ml streptomycin. MCF10A cells were maintained in a DMEM/Ham F12 mixture (3/1), supplemented with horse serum (Invitrogen), hydrocortisone (Sigma, St Louis, Missouri, USA), insulin (Sigma), EGF (Invitrogen) and choleratoxin.

2.7. Immunostaining and microscopy

Cells were grown on glass coverslips, washed with PBS and fixed with 3.7% paraformaldehyde for 20 min at room temperature. After permeabilization with 0.2% Triton X-100 in PBS, cells were blocked in 1% BSA in PBS and incubated at 37°C for 1 h, followed by Alexa Fluor-488/594 goat anti-mouse/rabbit IgG (Molecular Probes Eugene, Oregon, USA) for 30 min at room temperature. Stained cells were examined using a Zeiss Axioplan II epifluorescence microscope. Images were captured

**Fig. 2.** Direct interaction between TAZ and the first PDZ domain of ZO proteins. (A) Lysate from HEK293T cells overexpressing Myc-TAZ was incubated with GST fusions of the three single PDZ domains of ZO-1 and ZO-2 bound to glutathione beads. Coprecipitating proteins were separated by SDS–PAGE. Myc-tagged TAZ was identified by Western blot with a monoclonal anti-Myc antibody. (B) Incubation of recombinant His6-TAZ with GST fusions of the first PDZ domain of ZO-1 and ZO-2 bound to glutathione beads showing a direct interaction. PDZ2 of ZO-1 is included as a negative control. SN = supernatants.

**Fig. 3.** Endogenous TAZ partially colocalizes with endogenous ZO-2 in the nucleus. (A and B) MDCK cells stained for endogenous TAZ (red) and ZO-1 (A) or ZO-2 (B) (green). (C and D) MCF10A breast cells were stained for TAZ (red) and ZO-1 (C) or ZO-2 (D) (green). Size bar = 20 μm.
using a cooled CCD Axiocam Camera and Axiovision 4.4 software (Zeiss, Götingen, Germany).

2.8. Transient transcriptional activation assays

Supplementary data.

3. Results

3.1. TAZ associates with ZO-1 and ZO-2 through its C-terminal PDZ binding motif

We fused TAZ to GST and used this fusion protein as an affinity probe following cotransfection of GST–TAZ with ZO-1/2 in HEK293T cells. GFP-tagged ZO-1 and ZO-2 were specifically retained by GST–TAZ (Fig. 1A, asterisks). To ascertain if the PDZ binding region of TAZ was required for this interaction we used a TAZ construct that lacks 10 residues at the carboxy-terminus (GST–TAZD). Significantly, neither ZO-1 nor ZO-2 was retained by the TAZ mutant that lacked the PDZ binding motif. These findings indicate that the carboxy-terminal PDZ binding motif of TAZ is required for binding with ZO-1 and ZO-2.

We next investigated whether GST–TAZ could also interact with endogenous ZO-1 or ZO-2. GST, GST–TAZ WT and GST–TAZD were transfected in HEK293T followed by GST pull down and immunoblotting with anti-zonula occludens -1/-2 (ZO-1/ZO-2) antibodies (Fig. 1B). ZO-1 and ZO-2 signals were observed in lanes representing GST–TAZ WT, in contrast to GST and GST–TAZD. These data indicate that ZO proteins recognize the C-terminal PDZ binding motif of TAZ, most likely via one of their PDZ domains.

3.2. TAZ binds directly to the first PDZ domain of ZO-1 and ZO-2

ZO proteins contain 3 PDZ domains organized in tandem array. To determine which of these interact with TAZ, we transfected HEK293T cells with N-terminally myc-tagged TAZ and incubated the lysate with GST–PDZ1, GST–PDZ2 or GST–PDZ3 of ZO-1 and ZO-2 immobilized on glutathione beads. A clear signal was detected only in the lane of the PDZ1 domain of ZO-1 or ZO-2 (Fig. 2A). These results are consistent with a model in which the PDZ binding motif of TAZ interacts with the PDZ1 domain of the ZO-proteins. To test whether these proteins interact directly we incubated purified recombinant GST–PDZ1–ZO-1 and GST–PDZ1–ZO-2, bound to glutathione beads, with recombinant His6–TAZ present in an Escherichia coli lysate. His6–TAZ interacts with the PDZ1 domain of ZO-1 or ZO-2 but no interaction was observed with either GST or GST–PDZ2 of ZO-1 (Fig. 2B) indicating that TAZ directly interacts with the first PDZ domain of ZO-1 and -2.

3.3. Colocalization of ZO-1/ZO-2 with TAZ

Weak TAZ staining was observed in the cytoplasm in Madin–Darby canine kidney cells (MDCK) and MCF10A cells with more prominent localization in the nucleus (Fig. 3A–D, middle panel). ZO-1 was primarily concentrated at cell–cell contacts in MDCK cells as reported previously [15] (Fig. 3A and C, left panel). Weak cytoplasmic staining of ZO-1 was also observed in MCF10A cells but a nuclear localization could not be detected (Fig. 3C, left panel). This is in stark contrast to ZO-2 staining which displays a pronounced nuclear accumulation in MDCK cells (Fig. 3B, left panel) in addition to a cytosolic and plasma membrane staining pattern in MCF10A.

![Fig. 4. Inhibition of TAZ-dependent transcriptional activation by ZO-2.](image-url)

(A) HEK cells were transfected with a luciferase reporter construct and Gal4-fused TAZ, with or without ZO-2 WT or mutant ZO-2 lacking the first PDZ domain. ZO-2 reduces TAZ-mediated transcription activation in a dose-dependent manner. Mutant ZO-2, lacking the first PDZ domain, still shows inhibition but to a lesser extent. (B) HEK cells were transfected with a luciferase reporter fused to the Runx2-responsive osteocalcin promotor. Luciferase transcription is activated through transfection of TAZ. This activation is potently reduced when ZO-2 is co-expressed. Results are the mean ± S.D. from 3 separate experiments.
The nuclear staining pattern of TAZ and ZO-2 overlaps implying that both proteins partially colocalize in the nucleus. Interaction between TAZ and ZO proteins is likely to be context- and cell type dependent: CaCo-2 cells that were grown for more than a week to promote the formation of functional tight junctions [16] showed a strong colocalization between TAZ and ZO-1 at the plasma membrane (Supplementary Fig. 2A). The staining pattern of ZO-1 in sparsely seeded MDA-MB 231 cells was quite peculiar: ZO-1 was detected in the cytoplasm and occasionally also in the nucleus. Intriguingly, ZO-1 colocalized with TAZ at the leading edge of migrating MDA cells (Supplementary Fig. 2B). Since TAZ has been characterized as a nuclear transactivator and colocalized with ZO-2, we focused further experiments on ZO-2.

3.4. ZO-2 inhibits transactivation properties of TAZ

To investigate the effect of this interaction on the transactivation function of TAZ we used a gal4-fused TAZ construct in which TAZ was fused to the minimal gal4 DNA binding domain. TAZ-gal4 strongly stimulated luciferase activity (Fig. 4A) as shown before [17]. However, when gal4-TAZ was co-expressed with increasing amounts of ZO-2 we observed a dramatic dose-dependent decrease in gal4-dependent luciferase activity (Fig. 4A), indicating that ZO-2 inhibits transcription activation activity of TAZ. Of note, co-expression of TAZ with a ZO-2 deletion mutant that lacks the first PDZ domain elicited a much weaker inhibitory effect (Fig. 4A). In a second approach we used a luciferase reporter construct containing Runx2 response elements in the osteocalcin promoter (p6OSE2-luc). Expression of osteocalcin is dependent on the Runx2 transcription factor [18] and TAZ [2]. Cells transfected with the reporter plasmid and Runx2 showed a ~threefold increase in transcriptional activity over basal level (Fig. 4B). Cotransfection with TAZ resulted in a dramatic increase in transcriptional activity over basal levels. Coexpression with ZO-2 dramatically reduced activation to even lower levels than those obtained with a TAZ construct lacking the PDZ binding motif (Fig. 4B). Cotransfection of ZO-2 without the first PDZ domain was still able to inhibit transcriptional activity, although to a lesser extent than the wild type.

To investigate if the observed inhibition might be due to translocation of TAZ-induced by ZO-2, we transfected HEK293T cells with HA tagged ZO-2 and GST tagged TAZ. We observed that HA ZO-2 prevented nuclear translocation of TAZ concomitant with a redistribution to the cytosol (Fig. 5B). In cells that did not express the ZO-2 construct, GST TAZ localized to the nucleus as well (Fig. 5C). Localization of endogenous ZO-2/TAZ is shown in Fig. 5A. These findings indicate that ZO-2 can control the subcellular location of TAZ.

Since overexpressed ZO-2 leads to inhibition of TAZ-mediated transactivation we were curious to see if knocking down ZO-2 protein levels would result in stimulation of TAZ activity. Human embryonic kidney (HEK) cells transduced with a short hairpin targeting ZO-2 or a control short hairpin were used for this purpose (Supplementary Fig. 3B). Luciferase values did not differ significantly from control (Supplementary Fig. 3A).

4. Discussion

Our results indicate that the PDZ binding motif of TAZ is recognized by the first PDZ domain of ZO-1 and ZO-2. Transactivator
assays suggest an inhibitory role of ZO-2 on TAZ-induced reporter gene expression. ZO-2 down regulation did not provoke increased TAZ activity. Perhaps differences in transcription activation only surface in cells with complete silencing of ZO-2 instead of partial knockdown. In addition, other PDZ proteins like ZO-1 and MUPP1 might also be involved in regulation of TAZ. Since in Drosophila only one homologue of ZO proteins is expressed (Tamoun), it would be interesting to study the effect of knocking down this gene.

Both ZO-1 and -2 are able to interact with TAZ in a direct way in vitro but surprisingly, endogenous ZO proteins did not co-immunoprecipitate when endogenous ZO was pulled down. One possible explanation might be inadequacy of the antibodies to detect the complex of ZO and TAZ. Another explanation might be that coimmunoprecipitation experiments are prone to detect high affinity interactions [19]. Thus, the TAZ–ZO interaction might be of a transient nature. This observation is similar to leucocyte phototyrosine phosphatase (LCPTP), a tyrosine phosphatase that interacts with MAP kinases [20]. Only when LCPTP was overexpressed, a fraction of endogenous MAP kinases could be immunoprecipitated. Signal transduction events require low affinity interactions with sufficiently high off-rates [21], backing up the hypothesis of a transient interaction between ZO proteins and TAZ. These observations may also be context dependent because in HEK cells ZO-2 is not enriched in the nucleus unlike in MDCK cells. However, overexpression of ZO-2 resulted in a near depletion of nuclear TAZ in these cells.

Additional proteins may be required to reinforce the interaction between TAZ and ZO. The most straightforward class of proteins that could build up such a tripartite complex are transcription factors. c-Jun is an interesting candidate as a "bridging" protein since that could build up such a tripartite complex are transcription factors. c-Jun is an interesting candidate as a "bridging" protein since c-Jun is an interesting candidate as a "bridging" protein since it also comprises PPXY motifs endowing it to bind WW domain interactions with sufficiently high off-rates [21], backing up the hypothesis of a transient interaction between ZO proteins and TAZ. These observations may also be context dependent because in HEK cells ZO-2 is not enriched in the nucleus unlike in MDCK cells. However, overexpression of ZO-2 resulted in a near depletion of nuclear TAZ in these cells.

Additional proteins may be required to reinforce the interaction between TAZ and ZO. The most straightforward class of proteins that could build up such a tripartite complex are transcription factors. c-Jun is an interesting candidate as a "bridging" protein since it also comprises PPXY motifs endowing it to bind WW domain containing proteins. Altogether, networking between TAZ and ZO-1 and ZO-2 offers a promising target in the control of proliferation and apoptosis, epithelial–mesenchymal transition and migration capacity of cancer cells.

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Appendix A. Supplementary data


References