The Hippo pathway regulates the size of organs by controlling two opposing processes: proliferation and apoptosis. YAP2 (Yes kinase-associated protein 2), one of the three isoforms of YAP, is a WW domain-containing transcriptional co-activator that acts as the effector of the Hippo pathway in mammalian cells. In addition to WW domains, YAP2 has a PDZ-binding motif at its C-terminus. We reported previously that this motif was necessary for YAP2 localization in the nucleus and for promoting cell detachment and apoptosis. In the present study, we show that the tight junction protein ZO (zonula occludens)-2 uses its first PDZ domain to form a complex with YAP2. The endogenous ZO-2 and YAP2 proteins co-localize in the nucleus. We also found that ZO-2 facilitates the nuclear localization and pro-apoptotic function of YAP2, and that this activity of ZO-2 is PDZ-domain-dependent. The present paper is the first report on a PDZ-based nuclear translocation mechanism. Moreover, since the Hippo pathway acts as a tumour suppressor pathway, the YAP2–ZO-2 complex could represent a target for cancer therapy.

Key words: Hippo pathway, nuclear translocation, PDZ domain, Yes kinase-associated protein 2 (YAP2), zona occludens protein.

INTRODUCTION

YAP (Yes kinase-associated protein) is one of the effectors of the Hippo pathway that controls the size of organs by coordinating two opposing processes: proliferation and cell death [1]. Originally, the YAP gene was isolated by functional cloning, as Yes- and Src-kinase-binding protein [2]. Subsequently, YAP was shown to act as a transcriptional co-activator downstream of various signalling complexes [3–6]. However, the most compelling evidence for YAP function came from genetic analyses in Drosophila, which revealed an oncogene-like activity of YAP and its fly orthologue, Yki (Yorkie) [7].

There are three major isoforms of YAP, named YAP1, YAP2 and YAP2-L (YAP2-long), which are generated by differential splicing [4,8]. YAP1 contains one WW domain, YAP2 contains two WW domains and YAP2-L has a 16-amino-acid-long insert within its transcriptional activation domain [4]. WW domains are small protein modules known to mediate protein complexes by interaction with linear proline-rich peptide motifs in cognate proteins [9,10]. Apart from WW domains, YAP also contains a transcriptional activation domain and a C-terminally located PDZ-domain-binding motif. YAP has the ability to translocate from the cytoplasm to the nucleus, a process controlled by phosphorylation and also by WW- and PDZ-domain-mediated complexes with several cytoplasmic proteins [11–14].

We reported previously that when human embryonic HEK (human embryonic kidney)-293 cells were grown in medium containing 1% serum, YAP2, but not YAP1, was able to stabilize the pro-apoptotic protein p73 and thus promote cell apoptosis [13]. We also confirmed that the cytoplasmic localization of YAP2 was dependent on YAP2 phosphorylation at Ser127 by the kinase Lats1 (large tumour suppressor 1). We further showed that the PDZ-binding motif was required for promoting apoptosis in cells grown in low serum [14]. In addition, our results showed that the PDZ-binding motif was necessary for YAP2 localization in the nucleus, as a YAP2 mutant with a deleted PDZ-binding motif was found only in the cytoplasm. In summary, these recent results pointed to the existence of an unknown PDZ-domain-containing protein (or proteins), which could act as a shuttle, facilitating YAP2 translocation from the cytoplasm to the nucleus. In the present study we show that ZO (zonula occludens)-2, which associates with tight junctions, is one of the candidate proteins that control the localization of YAP2.

Among the prominent components of the tight junction-associated proteins are the ZO proteins ZO-1, -2 and -3. They belong to the MAGUK (membrane-associated guanylate kinase) protein family. Like other MAGUKs, the ZO proteins contain three N-terminally located PDZ domains, one SH3 (Src homology 3) domain, a GUK (guanylate kinase) homology domain and a proline-rich C-terminal region. All three proteins show extensive
sequence similarity to each other, especially within their equivalent PDZ domains [15]. Both ZO-2 and -3 can heterodimerize with ZO-1 through their second PDZ domain, but not with each other [16–19]. At tight junctions, ZO-1, -2, and -3 bind to claudins, JAM (junction adhesion molecule) and occludin [20–22]. They also bind actin filaments [19,23,24] and other signalling proteins, serving as putative adaptors in the assembly of signalling complexes [25]. However, the exact function of ZO proteins in the formation and maintenance of tight junctions is still unknown.

The subcellular distribution of ZO proteins seems to be sensitive to the degree of cell–cell contact [26,27]. Generally, ZO-1 and -2 concentrate in the nuclei of sparsely populated epithelial cells, whereas in confluent monolayers they tend to accumulate at tight junctions. This suggests that ZO proteins could transmit information about the state of cell–cell contact to the nucleus, and such signals could maintain balance between proliferation and differentiation. Both ZO-1 and ZO-2 proteins contain NLSs (nuclear localization signals) and NESs (nuclear export signals), but the function of these signals was verified only for ZO-2 [28,29]. Nuclear ZO-2 regulates gene expression through its association with various transcription factors including Jun, Fos and C/EBP (CCAAAT/enhancer-binding protein) [30]. ZO-1 has also been shown to regulate gene expression through the transcription factor CSA (cold-shock domain protein A; also known as ZONAB and DbpA). CSA stimulates cell proliferation in epithelial cells, and this process is negatively regulated by ZO-1, which sequesters the transcription factor at tight junctions [31,32].

The previous reports showing the involvement of YAP2 in the regulation of cell adhesion [13] and in cell-density-dependent nuclear localization of YAP [33] prompted us to study the function of the potential complex between YAP2 and ZO-2 proteins. We show in the present study that: (i) the complex between endogenous YAP2 and ZO-2 proteins could be visualized by immunostaining and co-immunoprecipitation; (ii) the PDZ-binding motif in YAP2 and the first PDZ domain in ZO-2 are required for the formation of the complex; (iii) the overexpression of ZO-2 affected the subcellular localization of YAP2; and (iv) that ZO-2 co-associates with YAP2 to enhance detachment of HEK-293 cells, but to inhibit YAP2-induced proliferation of MDCK (Madin–Darby canine kidney) cells. The novelty of the present work is in implicating the PDZ domain and the PDZ-domain-containing protein ZO-2 in the mechanism of nuclear transport.

**EXPERIMENTAL**

**Cell culture and transfections**

HEK-293 and MDCK cells were from the A.T.C.C. and cultured in DMEM (Dulbecco’s modified Eagle medium) with 10% (v/v) FBS (fetal bovine serum), MCF7 cells were cultured in Eagle’s minimum essential medium containing 10% (v/v) FBS and 10 μg/ml insulin (Sigma–Aldrich). MCF10A cells were cultured in MEGM (mammary epithelial cell growth medium) containing 10% (v/v) FBS, 50 μg/ml BPE (bovine pituitary extract), 0.5 μg/ml hydrocortisone (Sigma–Aldrich), 10 μg/ml insulin, 20 ng/ml EGF (epidermal growth factor; Sigma–Aldrich) and 0.1 μg/ml cholaer toxin (Calbiochem). HEK-293 cells were transfected using Lipofectamine™ (Invitrogen), according to the manufacturer’s instructions, and GenJet™ in vitro DNA transfection reagent (SignaGen) was used to transfect plasmids into MDCK cells.

**Antibodies and immunoprecipitation**

Anti-Flag-M2 antibody and anti-ZO-2 antibody were from Sigma–Aldrich and Santa Cruz Biotechnology respectively. Antibodies against ZO-1 and ZO-2 for immunostaining were from Invitrogen. Anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody was from Abcam and anti-PARP [poly(ADP-ribose) polymerase] antibody was from Roche Applied Science. The polyclonal anti-YAP antibody was generated in rabbits as described previously [8]. Immunoprecipitation was conducted as described previously [13,14]. To purify overexpressed Flag–YAP2 from HEK-293 cell lysates, 3 × Flag peptide (Sigma) was used according to the manufacturer’s instructions.

**Immunostaining**

Cultured cells were grown on glass coverslips, washed with PBS and fixed in 3% (w/v) paraformaldehyde. After permeabilization with 0.1% Triton X-100 in PBS, cells were blocked in 1% (w/v) BSA in PBS and incubated with primary antibody at 37°C for 1 h or at 4°C overnight, followed by Alexa Fluor® 594 goat anti-rabbit (for YAP) or Alexa Fluor® 488 anti-mouse (for ZO proteins) IgG (Molecular Probes) for 30 min at room temperature (25°C). Stained cells were examined using an ApoTome Zeiss Axioplan II epifluorescence microscope. Images were captured using a cooled CCD (charge-coupled-device) Axiocam Camera and Axiovision 4.4 software (Zeiss).

**Plasmids**

Human ZO-2 cDNA in pOTB7 vector was purchased from Open Biosystems. The amplified PCR product was subcloned into pBluescript vector (Stratagene) by utilizing KpnI and XbaI sites for PCR to construct primers that had been incorporated into the primers. This insert was subcloned into pEGFP-C3 vector (Clontech), p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vector (Invitrogen) using the KpnI and XbaI sites. ZO-2-N, a truncated fragment of ZO-2-WT (wild-type), was subcloned from pBluescript-ZO-2-WT into pEGFP-C3 vector, p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vector using the KpnI and BamHI/BglII sites. pcDNA4/TO/myc-His vector from the KpnI and BglII sites. ZO-2-WT into pEGFP-C3 vector, p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vector is 313–873 bp was subcloned into pBluescript-ZO-2-WT (wild-type), was subcloned into pBluescript-ZO-2-WT (wild-type), was subcloned from pBluescript-ZO-2-WT into pEGFP-C3 vector, p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vector from the KpnI and BglII sites. The resulting ZO-2 gene containing these mutations was synthesized by Epoch Bioslabs and the mutated fragment was exchanged with ZO-2-WT in p2 × Flag-CMV2 vector using the KpnI and BglII sites. The resulting plasmid was subcloned into the pEGFP-C3 vector and pcDNA4/TO/myc-His vectors from the KpnI and BamHI sites. The resulting plasmid was subcloned into the pEGFP-C3 vector, p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vectors from the KpnI and BglII sites. The resulting plasmid was subcloned into the pEGFP-C3 vector, p2 × Flag-CMV2 vector and pcDNA4/TO/myc-His vectors from the KpnI and BamHI sites. The resulting plasmid was subcloned into the pEGFP-C3 vector and pcDNA4/TO/myc-His vectors from the KpnI and BamHI sites. The resulting plasmid was subcloned into the pEGFP-C3 vector and pcDNA4/TO/myc-His vectors from the KpnI and BamHI sites. The resulting plasmid was subcloned into the pEGFP-C3 vector and pcDNA4/TO/myc-His vectors from the KpnI and BamHI sites. The resulting plasmid was subcloned into the pEGFP-C3 vector and pcDNA4/TO/myc-His vectors.
amplified fragments were inserted into pGEX4T3 vector to generate GST–human-ZO-2–1stPDZ-WT and GST-human-ZO-2–2ndPDZ–ΔNLS respectively.

Mouse ZO-2 cDNA was a gift from Dr S. Tsukita (Osaka University, Suita, Japan). Each of its three individual PDZ domains were cloned into the pGEX2TK vector using the BamHI and EcoRI sites (Amersham Pharmacia Biotech) and were named GST–1stPDZ, GST–2ndPDZ and GST–3rdPDZ respectively. The recombinant plasmids generated in the present study have been deposited in the Addgene plasmid repository (http://www.addgene.org). All other plasmids were as described previously [13,14].

Human ZO-1 cDNA in the pCR-Blunt II-TOPO vector was purchased from Open Biosystems. The amplified PCR product was subcloned into p2 × Flag-CMV2 vector in the same manner as for ZO-2.

GST-tagged protein expression and binding assay
The expression of GST-tagged proteins was induced by adding IPTG (isopropyl β-D-thiogalactopyranoside). The cells were harvested prior to adding Triton buffer [50 mM Tris/HCl, pH 7.45, 150 mM NaCl, 1 mM EDTA and 1% (v/v) Triton X-100]. Cell debris was removed by centrifugation (16 100 × g, 15 min), and glutathione–Sepharose 4B (Amersham Biosciences) was added to the supernatant and incubated for 1 h. The resin was washed with Triton buffer and then purified Flag–YAP2–WT from HEK-293 cell lysates was incubated with the resin for 2 h. The resin was washed again with Triton buffer and bound proteins were separated by SDS/PAGE followed by immunoblotting or Coomassie Blue staining.

RNAi (RNA interference) treatment and cell counting assay
The RNAi oligonucleotide against ZO-2 and the control oligonucleotide were purchased from Santa Cruz Biotechnology and Eurogentec. siRNA duplexes targeting canine ZO-2 were as follows: sense sequence, 5′-GCAGCAGUAUUCGCGACUAUdTdT-3′ and antisense sequence, 5′-AUAUGUCGGAUACUCGUCGdTdT-3′. MDCK cells were transfected with 50 nM siRNA duplexes using Lipofectamine 2000™ (Invitrogen) according to the manufacturer’s instructions. A negative control siRNA duplex, obtained from Eurogentec, was used under similar conditions. HEK-293 cells that express YAP2–WT in an inducible system [13,14] were plated at approx. 5% confluency in DMEM, 1% (v/v) FBS and 5 µg/ml blastidicin. Expression of YAP2 was induced by 1 µg/ml tetracycline. Transfections of the RNAi oligonucleotides were conducted using Lipofectamine™. After 96 h, floating cells were removed and attached cells were trypsinized and counted. For the cell counting assay without RNAi treatment, HEK-293 cells were transfected with Flag-tagged plasmids. After 24 h, the cells were distributed into new plates and maintained in DMEM with 1% (v/v) FBS for 96 h. After the removal of floating cells, attached cells were counted as described above. MDCK cells were maintained in DMEM with 0.5% FBS for 96 h. The expression of proteins was checked by Western blotting.

Establishment of MDCK and MCF10A cells that stably express YAP2 and ZO-2
MDCK cells were transfected with pBABE-puro-YAP2–WT, pBABE-puro-Flag-ZO-2 (–WT or –ANLS) or pBABE-puro control vector. Cells which failed to express these plasmids were removed by adding puromycin (Sigma–Aldrich) to a final concentration of 2 µg/ml. After the selection, cells were transfected with either pBABE-hygro or pBABE-hygro vectors containing ZO-2s. Selection was performed again by adding both 2 µg/ml puromycin and 400 µg/ml hygromycin (Invitrogen). Single cell isolation was never performed when cells were selected. The same procedure was performed for MCF10A cells except lower concentrations of puromycin (0.5 µg/ml) and hygromycin (200 µg/ml) were used, and pBABE-puro-YAP2–5SA was used instead of pBABE-puro-YAP2–WT.

RESULTS
Endogenous ZO-2 co-localizes with endogenous YAP in several cell lines
The C-terminal sequence of YAP (FLTWL) represents a well-conserved motif that binds to one of the 16 subclasses of PDZ domains [34,35]. By scanning billions of random peptides with 82 PDZ domains from human and Caenorhabditis elegans proteomes, a map of binding specificities was completed [35]. We used this map to predict several PDZ domains and their host proteins as putative partners of YAP. Four PDZ domain-containing proteins displayed a specificity profile that matched the C-terminus of YAP: the ZO family, MPDZ (multiple PDZ domain protein), SLC9A3R2 [solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2] and INADL (PDZ domain protein), SLC9A3R2 [solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2] and INADL (PDZ domain protein). In HEK-293 cells that express YAP2–WT from HEK-293 cell lysates was incubated with the resin for 2 h. The resin was washed again with Triton buffer and bound proteins were separated by SDS/PAGE followed by immunoblotting or Coomassie Blue staining.

The RNAi oligonucleotide against ZO-2 and the control oligonucleotide were purchased from Santa Cruz Biotechnology and Eurogentec. siRNA duplexes targeting canine ZO-2 were as follows: sense sequence, 5′-GCAGCAGUAUUCGCGACUAUdTdT-3′ and antisense sequence, 5′-AUAUGUCGGAUACUCGUCGdTdT-3′. MDCK cells were transfected with 50 nM siRNA duplexes using Lipofectamine 2000™ (Invitrogen) according to the manufacturer’s instructions. A negative control siRNA duplex, obtained from Eurogentec, was used under similar conditions. HEK-293 cells that express YAP2–WT in an inducible system [13,14] were plated at approx. 5% confluency in DMEM, 1% (v/v) FBS and 5 µg/ml blastidicin. Expression of YAP2 was induced by 1 µg/ml tetracycline. Transfections of the RNAi oligonucleotides were conducted using Lipofectamine™. After 96 h, floating cells were removed and attached cells were trypsinized and counted. For the cell counting assay without RNAi treatment, HEK-293 cells were transfected with Flag-tagged plasmids. After 24 h, the cells were distributed into new plates and maintained in DMEM with 1% (v/v) FBS for 96 h. After the removal of floating cells, attached cells were counted as described above. MDCK cells were maintained in DMEM with 0.5% FBS for 96 h. The expression of proteins was checked by Western blotting.

Establishment of MDCK and MCF10A cells that stably express YAP2 and ZO-2
MDCK cells were transfected with pBABE-puro-YAP2–WT, pBABE-puro-Flag-ZO-2 (–WT or –ANLS) or pBABE-puro control vector. Cells which failed to express these plasmids were removed by adding puromycin (Sigma–Aldrich) to a final concentration of 2 µg/ml. After the selection, cells were transfected with either pBABE-hygro or pBABE-hygro vectors containing ZO-2s. Selection was performed again by adding both 2 µg/ml puromycin and 400 µg/ml hygromycin (Invitrogen). Single cell isolation was never performed when cells were selected. The same procedure was performed for MCF10A cells except lower concentrations of puromycin (0.5 µg/ml) and hygromycin (200 µg/ml) were used, and pBABE-puro-YAP2–5SA was used instead of pBABE-puro-YAP2–WT.
Figure 1 Endogenous ZO-2 co-localizes with endogenous YAP in MDCK cells

(A) Endogenous ZO-1, ZO-2 and YAP2 in MDCK cells were immunostained and observed with a microscope. Scale bar, 20 µm. (B) Endogenous ZO-2 and YAP2 in indicated cell lines were immunostained and observed with a microscope. Scale bar, 20 µm. (C) ZO-1, ZO-2 and YAP2 form a complex. Flag–ZO-1-WT, Myc–His–ZO-2-WT and Myc–His–YAP2-WT were co-transfected into HEK-293 cells. Cell lysates were immunoprecipitated (IP) with either control IgG or an anti-Flag antibody, resolved on SDS gels and immunoblotted (IB) with the indicated antibodies. Molecular masses (MW) are indicated in kDa on the left-hand side. (D) Endogenous ZO-2 binds to endogenous YAP in MDCK cells. MDCK cells were maintained in two different conditions. One was very sparsely distributed and the other was very dense (overgrowth). To keep the cells healthy, the medium was exchanged every 24 h for 5 days after the cells had reached confluence. The same amount of MDCK cell lysates were used for immunoprecipitation. The cell lysates were immunoprecipitated with either control IgG or an anti-YAP antibody, resolved on SDS gels and immunoblotted with anti-ZO-2 or anti-YAP antibodies. Molecular masses are indicated in kDa on the left-hand side. (E) Endogenous ZO-2 binds to endogenous YAP in both cytosol and nucleus of MDCK cells. Cytosolic and nuclear fractions of MDCK cells were separated by using CelLytic™ NuCLEAR™ extraction kit (Sigma–Aldrich). These fractions were immunoprecipitated with either control IgG or anti-YAP antibody, resolved on SDS gels and immunoblotted with indicated antibodies. Molecular masses are indicated in kDa on the left-hand side. (F) Both YAP and ZO-2 were slightly increased in the nucleus when MDCK cells were sparsely distributed. MDCK cells were maintained as described in (D), and cytosolic and nuclear fractions were separated as described in (E). A total of 50 µg of extracts was run on each lane, followed by immunoblotting with indicated antibodies to monitor the distribution of endogenous proteins. Molecular masses are indicated in kDa on the left-hand side.
signal between the input and the immunoprecipitated fraction, ZO-1 binds to ZO-2 more tightly than to YAP2. To test whether the association of YAP and ZO-2 changes depending on the state of cell confluency, MDCK cells were maintained in sparse or dense conditions; however, consistently, we did not detect significant changes in the YAP2–ZO-2 complex as a function of cell density (Figure 1D). The association of endogenous ZO-2 and YAP in the nucleus and cytosol was confirmed when immunoprecipitation was conducted using nuclear and cytosol fractions of MDCK cells (Figure 1E). It was shown previously that ZO-2 accumulated in the nucleus when MDCK cells were sparsely distributed, whereas when the cells were densely distributed ZO-2 could be also found in the cytoplasm [27]. To investigate whether localization of ZO-2 is accompanied by that of YAP in MDCK cells, nuclear and cytosol fractions of MDCK cells were isolated and the distribution of endogenous ZO-2 and YAP was monitored. However, we observed a less robust change compared with the results reported previously [27]. Nevertheless, we re-confirmed that the ratio of ZO-2 in the nucleus compared with the cytoplasm increased when MDCK cells were sparsely distributed. As observed for ZO-2, a similar change in the subcellular distribution was observed for YAP2 (Figure 1F). The amounts of ZO-2 and YAP in the nucleus slightly decreased when MDCK cells were densely populated (Figure 1F). On the basis of these observations, we decided to investigate further the role of the ZO-2–YAP association in mammalian cells.

YAP2 PDZ-binding motif binds the first PDZ domain of ZO-2

Because ZO-2 has three PDZ domains and YAP2 has a PDZ-binding motif at its C-terminus, we hypothesized that ZO-2 associates with YAP2 via its PDZ domain(s). From the comprehensive map of PDZ-binding specificities [35] we predicted that only the first PDZ domain of ZO-2 would bind YAP2. The first, second and third PDZ domains of mouse ZO-2 were individually expressed as GST-fused domains and used to pull down Flag–YAP2 from HEK-293 cells (Figure 2A). As expected, only the first PDZ domain of ZO-2 pulled down YAP2. Using ITC (isothermal titration calorimetry) we determined that the dissociation constant $K_d$ for the 11-mer peptide of YAP (KLDKESFLTWL) and the GST–PDZ1 of ZO-2 was 2.3 μM. A control peptide with a deletion of the last three amino acids (-TWL) did not result in thermodynamic changes in the ITC assay (results not shown). Therefore we concluded that the truncated peptide probably did not interact with the PDZ domain. Interestingly, the PDZ1 domain of ZO-1 interacted with the YAP peptide with similar strength ($K_c = 3.7$ μM) (Figure 2B).

To study the function of the ZO-2–YAP2 complex in detail, several ZO-2 mutants were constructed. Both ZO-1 and ZO-2 were shown previously to form a complex with ARVCF (armadillo-repeats gene deleted in velocardiofacial syndrome) protein, and their PDZ domains were responsible for the association [39]. In that report, four point mutations within the binding pocket of the first PDZ domain of ZO-1 were shown to be required to abolish the binding. The equivalents of the ZO-1 PDZ domain mutations were introduced into the ZO-2 PDZ1 domain (K38E, F44H, I46E and V48E) (see construct ZO-2-PDZm in Figure 3A and Supplementary Figure S3 at http://www.BiochemJ.org/bj/432/bj4320461add.htm). Since we showed that the first PDZ domain bound YAP2, a ZO-2,-ΔN mutant that lacks the first PDZ domain was constructed. We also generated a mutant of ZO-2 in which two putative NLSs [21,29,38] were eliminated (ZO-2,-ΔNLS). A truncated version of ZO-2 that has only the first PDZ domain (ZO-2-N) was also constructed. Binding assays were conducted with these mutants. ZO-2-WT could co-precipitate YAP2 effectively (Figure 3B). This binding between ZO-2 and YAP2 was weakened significantly when the first PDZ domain of ZO-2 was impaired by the four point mutations (Figure 3B). The binding of ZO-2-N to YAP2 was as strong as ZO-2-WT, and this binding signal was stronger than those of the ZO-2-PDZm,-ΔN or -ΔNLS mutants, suggesting again that the first PDZ domain of ZO-2 associates with YAP2.

On the basis of the map of PDZ domain-binding specificities [35] we hypothesized that the complex between YAP2 and ZO-2 is mediated mainly, if not exclusively, by the first PDZ domain. To test this hypothesis, we used a ΔC mutant of YAP2 lacking the five C-terminal residues [14], which constitute the PDZ-binding motif (Figure 3C). Flag-tagged YAP2-WT, YAP2,-ΔC and control vector were transfected into HEK-293 cells followed by immunoprecipitation and immunoblotting. YAP2-WT bound strongly to endogenous ZO-2, whereas binding between YAP2-ΔC and the ZO-2 protein was barely detectable, suggesting that the PDZ-binding motif in YAP2 is necessary to form the complex with ZO-2 (Figure 3D). Taken together, our results indicate...
that binding between YAP2 and ZO-2 is mediated mainly by the PDZ-binding motif of YAP2 and the first PDZ domain of ZO-2.

**YAP2 localization is regulated by ZO-2**

We showed that the endogenous YAP2 and ZO-2 proteins co-localize in the nucleus of several cell lines (Figure 1), and both ZO-2-WT and ZO-2-ΔNLS bind to YAP2, although ZO-2-WT bound to YAP2 more tightly than ZO-2-ΔNLS (Figure 3B). To test whether ZO-2-WT and ZO-2-ΔNLS have any influence on YAP2 localization in MDCK cells, we established MDCK cells stably expressing ZO-2-WT, ZO-2-ΔNLS or control vector. ZO-2-WT was distributed in both the cytosol and nucleus, but the stronger signal was detected in the nuclear fraction. On the other hand, ZO-2-ΔNLS was detected only in the cytosolic fraction (Figure 4A, top panel). Interestingly, distribution of endogenous YAP in the nucleus consistently increased when ZO-2-WT was expressed and it significantly decreased when ZO-2-ΔNLS was present (Figure 4A, middle panel, lanes 2, 4 and 6). Similar results were obtained in HEK-293 cells (Supplementary Figure S4 at http://www.BiochemJ.org/bj/432/bj4320461add.htm). These results support our assumption that ZO-2 regulates the distribution of YAP in cells. To confirm this assumption further, GFP (green fluorescent protein)–ZO-2-WT or GFP–ZO-2-ΔNLS were individually co-transfected with YAP2 tagged with the red fluorescent protein DsRed into MDCK cells and their localizations were observed (Figures 4B and 4C). As we had expected, ZO-2-ΔNLS was generally excluded from the nucleus, whereas ZO-2-WT was predominantly nuclear in MDCK cells. GFP–ZO-2-WT co-localized with DsRed–YAP2-WT in the nucleus as well as in the cytosol (Figure 4B). It was reassuring to see the same locale for both GFP-tagged ZO-2 and YAP2 as that observed for the endogenous proteins (Figure 1). The percentage of cells in which DsRed–YAP2-WT was observed in the nucleus was more than 75 % when GFP–ZO-2-WT was present (Figure 4C). The percentage of cells in which DsRed–YAP2-WT was observed in the nucleus was significantly decreased to 11.2 % when GFP–ZO-2-ΔNLS was present (Figure 4C). These results suggest that ZO-2 can regulate the subcellular localization of YAP2.

To make sure that by generating the ZO-2-ΔNLS deletion mutant we did not affect the neighbouring PDZ domain, two binding assays were conducted. Since ZO-2 forms homodimers as well as heterodimers with ZO-1 via the second PDZ domain [19,40–42], we verified that both GFP–ZO-2-WT and GFP–ZO-2-ΔNLS were precipitated by Flag–ZO-2-WT (Figure 4D). These results suggest that our ZO-2-ΔNLS construct was still capable of binding to ZO-2. Next, the ability to bind to YAP2 was tested to rule out the possibility that ZO-2-ΔNLS fails to bind YAP2 because its first PDZ domain is not intact. The ZO-2 first PDZ domain is responsible for the association with YAP2 (Figure 2A) and the domain consists of 85 amino acids; in the ZO-2-ΔNLS construct the PDZ1 domain lacks 13 amino acids at the C-terminus (see the orange brackets in Supplementary Figure S3A). Although notably weaker than with the WT-1stPDZ domain, GST-tagged
Function of the first PDZ domain of ZO-2 and YAP

Figure 4  Co-localization of YAP2 and ZO-2

(A) YAP2 accumulates in the nucleus in the presence of ZO-2-WT. Cytosol and nuclear fractions of MDCK cells stably expressing Flag–ZO-2-WT, Flag–ZO-2-ΔNLS or control vector were separated by using the CelLytic<sup>TM</sup> NuCLEAR<sup>TM</sup> extraction kit, followed by immunoblotting (IB) with indicated antibodies. Molecular masses (MW) are indicated in kDa on the left. (B) Effects of ZO-2 on the localization of YAP2. DsRed–YAP2-WT was co-transfected with GFP–ZO-2-WT (upper panels) or GFP–ZO-2-ΔNLS (lower panels) in MDCK cells. The localization of each protein was observed. The nucleus was stained with DAPI (4′,6-diamidino-2-phenylindole). Scale bar, 20 µm. (C) Graphic representation of results shown in (B). The ratios of nuclear localization of DsRed–YAP2-WT in the presence of GFP–ZO-2-WT or GFP–ZO-2-ΔNLS are indicated. If the red or green signals overlapped with blue signal of DAPI, the protein was defined as ‘localized in the nucleus’. Cells expressing both GFP and DsRed constructs were counted, and cells expressing only one of them were eliminated from this evaluation. The experiment was repeated three times independently and approx. 100 cells were observed in each case. (D) ZO-2-ΔNLS does not lose the ability to form a homodimer. The indicated plasmids were co-transfected into HEK-293 cells. Cell lysates were immunoprecipitated (IP) with anti-Flag antibodies, resolved via SDS/PAGE, and immunoblotted (IB) with anti-GFP antibody (top panel). The middle and bottom panels show the expression of transfected proteins. Molecular masses (MW) are indicated in kDa on the left. (E) ZO-2-ΔNLS does not lose the ability to bind to YAP2. The indicated portions of human ZO-2 were individually fused to GST. GST–1stPDZ-WT contains residues 1–303 of human ZO-2; GST–1stPDZ-ΔNLS contains residues 1–104 and 292–303, and GST–3rdPDZ contains residues 501–600 amino acids. GST-tagged proteins were purified with glutathione–Sepharose 4B followed by incubation with purified Flag–YAP2 by using anti-Flag M2 affinity gel (Sigma–Aldrich) and 3 × Flag peptide. Bound proteins were separated by SDS/PAGE followed by immunoblotting or Coomassie Blue staining. Molecular masses are indicated in kDa on the left-hand side.

ZO-2–1stPDZ–ΔNLS was able to bind to YAP2 in vitro (Figure 4E and also see Figure 3B).

To confirm further that GFP–ZO-2 functions with YAP2 in the nucleus, we used the YAP2–RUNX2 (runt-related transcription factor 2) transactivation assay of the osteocalcin gene, which was originally reported as the functional ‘read-out’ of YAP [6]. Increasing amounts of GFP–ZO-2 had an inhibitory effect on the transactivation function of YAP2–RUNX2 for the osteocalcin gene luciferase reporter (Supplementary Figure S5 at http://www.BiochemJ.org/bj/432/bj4320461add.htm).
We previously reported that YAP2 promotes cell detachment and apoptosis in HEK-293 cells [13], and that this function was impaired when the PDZ-binding motif of YAP2 was deleted [14]. Since we detected the binding between the PDZ-binding motif of YAP2 and the first PDZ domain of ZO-2 (Figure 3), and YAP2 accumulated in the nucleus when ZO-2 was co-transfected (Figure 4), we hypothesized that ZO-2 regulated the function of YAP2 in terms of cell detachment. To investigate this assumption, we down-regulated the level of ZO-2 by RNAi in HEK-293 cells and monitored changes in YAP2-induced cell detachment. The expression of YAP2-WT was induced in HEK-293 cells followed by transfection of either ZO-2-specific RNAi oligonucleotides or control RNAi oligonucleotides. The efficiency of this RNAi oligonucleotide was confirmed by immunoblotting and immunostaining (Figures 5A and 5B), and it was able to reduce the amount of ZO-2 by more than 99%.

After 96 h of culture in DMEM containing 1% (v/v) serum, the attached cells were counted. When the control RNAi was transfected, the ratio of attached cells with induced YAP2-WT to attached cells without induced YAP2-WT was 0.495 (Figure 5A). However, when ZO-2 RNAi was transfected, the ratio was increased to 0.650 (Figure 5A). The amount of the cleaved PARP fragment was decreased when endogenous ZO-2 was down-regulated (Figure 5A, bottom panel, lanes 2 and 4), suggesting that apoptosis was repressed. In addition, the amount of YAP in the nucleus of HEK-293 cells was decreased by 37.2% when ZO-2 was removed (Figure 5C). These results indicate that the ability of YAP2 to localize in the nucleus and promote cell detachment and apoptosis in HEK-293 cells was partially impaired when the level of ZO-2 protein was diminished.

We conducted a comparable assay with MDCK cells. However, unlike HEK-293 cells, the suppression of ZO-2 in MDCK cells was never complete in our hands, but only partial, which might be a reason why ZO-2 RNAi treatment had no influence on YAP localization in MDCK cells (Supplementary Figure S6 at http://www.BiochemJ.org/bj/432/bj4320461add.htm).

ZO-2 protein expression enhances YAP2 ability to promote cell detachment

When endogenous ZO-2 was removed, the ability of YAP2 to promote cell detachment was decreased (Figure 5A). This result prompted us to test whether the overexpression of ZO-2-WT (as well as of several ZO-2 mutants) had any effect...
on the function of YAP2. YAP2 was transfected with several ZO-2 constructs into HEK-293 cells. At 24 h after transfection, cells were trypsinized and plated at the same density (25000 cells per plate). After 4 days of culture the number of cells was counted (Figure 6A). The number of cells transfected with vector alone increased 16.4-fold. This number of cells in which YAP2 and the vector were transfected increased to a lesser level than control, namely 11.6-fold, in agreement with our previous findings [13,14]. Interestingly, the number of cells in which ZO-2 was co-transfected with YAP2 increased by an even lower level (9.4-fold). ZO-2-N, a mutant localized in the nucleus, showed a slightly stronger inhibitory effect (8.8-fold) than ZO-2-WT. Other mutants (ZO-2-PDZm, -ΔN and -ΔNLS) had minor effects on YAP2 function to reduce cell proliferation. This might be due to their weak association with YAP2 (Figure 3). In contrast with ZO-2, ZO-1-WT did not enhance the ability of YAP2 to promote cell detachment. PARP cleavage was clearly detectable, especially when ZO-2-WT or ZO-2-N was overexpressed.

To determine the effects of YAP2 and ZO-2 in other cells, MDCK and MCF10A cells stably expressing YAP2 and ZO-2 were established. In contrast with HEK-293 cells, YAP2 promotes cell proliferation in MDCK cells. This effect was abrogated when ZO-2-WT was co-expressed (Figure 6B). This result is consistent with previous findings that suggested that ZO-2 in some settings could act as a tumour suppressor gene [43,44]. ZO-2 mutants slightly diminished the pro-proliferative effects of YAP2, but these results were not statistically significant (P > 0.05). For unknown reasons, the expression of ZO-2-N was consistently weaker than that of other ZO-2 constructs in these MDCK cells (results not shown). In MCF10A cells, it was shown that YAP2 induced EMT (epithelial-to-mesenchymal transition) [45]. Therefore we decided to see whether ZO-2 had any effect on EMT in MCF10A cells. In positive and negative control experiments, we reproduced EMT in MCF10A cells, but the overexpression of ZO-2 had little or no influence on EMT (results not shown).

Our results suggest that ZO-2 co-operates with YAP2 to promote cell detachment and apoptosis in HEK-293 cells. However, in MDCK cells, YAP2 promotes cell proliferation whereas ZO-2 prevents it. Even though YAP2 accumulates in the nucleus in the presence of ZO-2, the anti-proliferative effects of ZO-2 are more dominant than pro-proliferative effects of YAP2 in MDCK cells. Identification of signalling partners downstream of the YAP2–ZO-2 complex in both cell types will help explain the functional duality of YAP, which can act either as an oncogene or a pro-apoptotic factor.

**DISCUSSION**

The Hippo signalling pathway was delineated as a string of signalling complexes that involve several tumour suppressors, and at least two effectors: YAP and TAZ (transcriptional co-activator with PDZ-binding motif) [also known as WWTR1 (WW-domain-containing transcription regulator 1)], which display oncogene-like activity [46,47]. When not affected by genetic lesions, the components of this pathway control the intrinsic size of organs by regulating the balance between proliferation and cell death. Therefore a detailed understanding of the molecular mechanisms that govern this pathway should help us better understand normal growth and its abnormal forms, including cancer.

In the present study, we elucidated a new aspect of the mammalian Hippo pathway, namely the signalling by a YAP2–ZO-2 complex that is mediated by the PDZ domain of ZO-2. We showed that the tight junction protein ZO-2 uses its first PDZ domain to form a complex with YAP2. We also showed that endogenous ZO-2 and YAP2 co-localize in the nucleus, and that ZO-2 regulates nuclear localization and the pro-apoptotic function of YAP2 in HEK-293 cells, and inhibits YAP2-induced proliferation in MDCK cells. Since the Hippo pathway is activated by cell-to-cell contacts [33], the identification of a member of the ZO family of tight junction proteins as a partner of YAP signalling adds further to the quickly expanding characterization of the Hippo signalling network [47].

The following aspects of the present study deserve further comment: (i) the role of ZO-2 among other ZO family members as the ‘preferred’ partner of YAP2; (ii) the cell-density-dependent localization of ZO-2 and YAP2 in cells; (iii) the functional implication of the absence of the PDZ-domain-binding region in the Drosophila YAP homologue Yki; (iv) targeting YAP complexes with PDZ-domain-containing...
proteins for cancer therapy; and (v) the role of YAP paralogue TAZ/WWTR1 in assembling functional complexes with ZO proteins.

We focused our study on ZO-2 as the partner of YAP2 because, unlike ZO-1, ZO-2 co-localized with YAP2 in the nuclei of kidney epithelial cells. In addition, co-immunoprecipitations showed a robust complex between ZO-2 and YAP2 in several cell lines. In contrast with ZO-1 and ZO-2, ZO-3 has not been reported in the nucleus, where YAP2 functions as a transcriptional co-activator. We anticipate that all three ZO proteins have a propensity to form complexes with YAP2 via their first PDZ domains. Such complexes could function in specific locales, in specific cells and under various physiological conditions. For example, ZO-2 is generally not expressed in non-epithelial cells, whereas ZO-1 is expressed and therefore it could ‘replace’ the ZO-2 function.

It has been shown that at low-cell-density YAP is unphosphorylated and nuclear. However, when cells reach the confluent state, YAP is phosphorylated and mainly cytoplasmic [33]. Interestingly, the subcellular distribution of ZO-1 and ZO-2 proteins is similar to YAP. In epithelial cells cultured in sparse conditions, ZO-1 and ZO-2 are nuclear. However, in a confluent state, both these ZO proteins accumulate in tight junctions [26,27]. However, we do observe that in some cell lines, including MDCK cells, a certain amount of YAP and ZO-2 stay concentrated in the nuclei, even after the cells were kept in a confluent state for extended periods of time. Nuclear phospholipids may be a contributing factor in this observation. PtdIns(4,5)P₂ is known to reside in nuclear spockles of MDCK cells and a recent study has shown that ZO-2 interacts with PtdIns(4,5)P₂ via its second PDZ domain [48]. Moreover, in that study ZO-2 was shown to co-localize with nuclear PtdIns(4,5)P₂. The possibility of ZO-2 forming a tripartite complex with YAP and PtdIns(4,5)P₂ and the regulatory role of PtdIns(4,5)P₂ on the stability or function of the nuclear YAP–ZO-2 complex remains the subject of further study.

Apparently, cells that harbour a mutation in Merlin, a gene that encodes one of the cytoplasmic, and possibly also nuclear component, [49] of the Hippo pathway, have YAP localized in the nuclei even in high-density cultures [50]. We assessed whether Merlin was mutated in our stock of MDCK cells, by amplifying its cDNA and performing sequence analysis and the analysed sequence of Merlin was identical with that of the WT dog Merlin cDNA (C. Gaffney, T. Oka and M. Sudol, unpublished work). It is possible that a mutation in other upstream components of the Hippo pathway could be responsible for the enhanced and cell-density-independent nuclear localization of YAP in MDCK cells.

In the case of the Drosophila YAP homologue Yki, the C-terminal sequence does not contain a known PDZ-binding sequence and there is no obvious internal PDZ ligand motif either [14]. Therefore the signalling of the mammalian YAP in the Hippo pathway differs in that particular aspect from the signalling by Yki in the fly. Moreover, the fly seems to be an exception, as the PDZ-binding motif is well-conserved in YAP orthologues throughout the animal kingdom. This is true even in simple Hydra, whose divergence is estimated to have occurred approx. 1 billion years ago (Supplementary Figure S7 at http://www.BiochemJ.org/bj/432/bj4320461add.htm) [14]. We suggest that Drosophila Yki must interact with other partner(s) that act similarly to mammalian ZO-2 protein, regulating the nuclear transport of Yki and sensing cell-to-cell contacts.

The loss of main components of the Hippo pathway leads to uncontrolled cell growth, which confirms that this is a tumour suppressor pathway. YAP, as the effector of the pathway, has been proposed to act as both an oncogene [51] and tumour suppressor [52]. The study of YAP expression in common solid tumours revealed relative changes in the subcellular localization of YAP in cancer cells compared with the normal controls [53]. Increases in both cytoplasmic and nuclear localization of YAP protein was generally observed in these tumours. Since YAP is a nuclear effector of the Hippo pathway [7,13], controlling its translocation to the nucleus, by interfering with the PDZ-domain-mediated complex(es) through small molecular inhibitors, one should be able to control the growth of cancer cells. This approach could be feasible since the -TWL sequence is present in the C-terminus of only three human proteins, and the C-terminus of YAP was not found to bind to other PDZ domains. Apart from YAP, and its close parologue TAZ, the -TWL end is also present in MRPL43 (mitochondrial ribosomal protein L43), suggesting that peptides or mimetics of this sequence could be specific as drugs, with possible minor side effects, as such drugs would not significantly affect signalling by other PDZ domain complexes.

Two comments are appropriate here. Because regulatory interactions are mostly co-operative, and regulatory decisions in signalling are generally made by large multi-component complexes and not by single proteins, or simple pair of proteins [54], we have to consider that there could be other PDZ domain-containing proteins, outside of the ZO family, which could also interact with YAP2 and contribute to the global regulation of YAP2 nuclear transport. Such regulations are detectable by signalling studies using sensitive ‘read-outs’, as shown by our analyses, but could not always be easily delineated by genetic studies because of the inherent redundancy.

In a separate study, we most recently documented that the YAP paralogue TAZ (WWTR1) also interacts with ZO-2 via the PDZ domain, co-localizes in the nucleus in various cells and affects transcription of a TAZ-regulated gene [55].

Our work implicates the PDZ domain and PDZ domain-containing protein ZO-2 in the mechanism of nuclear translocation of YAP protein, one of the main effectors of the Hippo tumour suppressor pathway.

ACKNOWLEDGEMENTS

We thank our colleagues L. Derte, C. Gaffney, J. Kissil and W. Schwindinger for comments on the manuscript, and Dr S. Tsukita and Dr X. Yang for DNA constructs. We thank C. Gaffney for help with sequencing of the cDNA of dog Merlin.

REFERENCES

Sudol, M. (1994) Yes-associated protein (YAP) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. Oncogene 9, 2145–2152


The Authors Journal compilation © 2010 Biochemical Society

Function of the first PDZ domain of ZO-2 and YAP


SUPPLEMENTARY ONLINE DATA

Functional complexes between YAP2 and ZO-2 are PDZ domain-dependent, and regulate YAP2 nuclear localization and signalling

Tsutomu OKA*, Eline REMUE†‡, Kris MEERSCHAERT†‡, Berlinda VANLOO†‡, Ciska BOUCHERIE†‡, David GFELLER§, Gary D. BADER§, Sachdev S. SIDHU§, Joël VANDEKERCKHOVE†‡, Jan GETTEMANS†‡ and Marius SUDOL*∥1

*Weis Center for Research, 100 North Academy Avenue, Danville, PA 17822, U.S.A., †Department of Medical Protein Research, VIB, Ghent University, B-9000 Ghent, Belgium, ‡Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Albert Baertsoenkaai 3, B-9000 Ghent, Belgium, §Terrence Donnelly Center for Cellular and Biomolecular Research, University of Toronto, 160 College Street, Toronto, Ontario, Canada, M5S 3E1, and ∥Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

Figure S1  PDZ domains and their host proteins that interact with human YAP

Five PDZ domains were predicted and tested with the human YAP sequence. The middle column displays the sequence logo derived from phage peptides [1]. The right-hand column shows the YAP C-terminus. Amino acids that clearly match the logo are highlighted in red.

1 To whom correspondence should be addressed (email msudol1@geisinger.edu).
**Figure S2** Molecular structures of PDZ-domain-containing proteins that also have NLSs

NLSs were predicted using the PredictNLS software [2]. Non-canonical NLS was also predicted [3]. Modular structure of proteins was taken from SMART [4]. The green rectangle indicates the localization of NLS within a given protein and the exact position of the first amino acid of the NLS and its sequence are shown in the two right-hand rows. Accession numbers refer to PubMed Gene IDs. ANK, ankyrin; CH, calponin homology; DAX, domain present in Dishevelled and axin; DEP, domain present in Dishevelled, Egl-10 and pleckstrin; Fha, forkhead-associated; GuKc, GUK; L27, ribosomal L27; LIM, zinc-binding domain present in Lin-11, Isl-1 and Mec-3; PH, pleckstrin homology; PTB, phospho-tyrosine binding; RA, RAS-association; RGS, RGS-box; RhoGAP, Rho GTPase-activating protein; SAM, sterile alpha motif; TCK-S, serine/threonine kinase; TK, tyrosine kinase.

<table>
<thead>
<tr>
<th>Accession Number</th>
<th>Modular structure</th>
<th>NLS position</th>
<th>Predicted NLS sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARB3</td>
<td>PDB-NLS-PDZ-PDZ</td>
<td>368</td>
<td>RRRRRRPQ</td>
</tr>
<tr>
<td>ARMSAP23</td>
<td>PDB-PY-PY-PY-PDZ</td>
<td>1076</td>
<td>WAWKRRRKE</td>
</tr>
<tr>
<td>DVL1</td>
<td>DAX-NLS-PDZ-OBP</td>
<td>216</td>
<td>BICKKRRRQGQK</td>
</tr>
<tr>
<td>DVL2</td>
<td>DAX-NLS-PDZ-OBP</td>
<td>192 / 235</td>
<td>KRRPKRR / KRRPKRRK</td>
</tr>
<tr>
<td>DVL3</td>
<td>DAX-NLS-PDZ-OBP</td>
<td>213</td>
<td>WRRKRRRQFQ</td>
</tr>
<tr>
<td>GSK2</td>
<td>61652</td>
<td>523</td>
<td>RICKRRR</td>
</tr>
<tr>
<td>LIMD7</td>
<td>CH-PDZ-NLS-LIM</td>
<td>655</td>
<td>LAYLULR</td>
</tr>
<tr>
<td>MAG21</td>
<td>PDZ-Glu-WW-WW-PDZ</td>
<td>1382</td>
<td>KRRRRRRRRR</td>
</tr>
<tr>
<td>MAG22</td>
<td>NLS-PDZ-Glu-WW-WW</td>
<td>5</td>
<td>KKKSK</td>
</tr>
<tr>
<td>MAG24</td>
<td>NLS-S,T14,S,T15,</td>
<td>560</td>
<td>KRRRRRRR</td>
</tr>
<tr>
<td>HMM5</td>
<td>L27-L27-PDZ-NLS</td>
<td>438</td>
<td>WRRKRRRQFQ</td>
</tr>
<tr>
<td>POZ2D2</td>
<td>PDZ-PDZ-PDZ-PDZ</td>
<td>104</td>
<td>KRRRRRRR</td>
</tr>
<tr>
<td>POZ2D4</td>
<td>PDZ-NLS</td>
<td>691</td>
<td>KRRRRRRR</td>
</tr>
<tr>
<td>PDZ2N3</td>
<td>RING-PDZ-PDZ-NLS</td>
<td>968</td>
<td>KRRRRRRR</td>
</tr>
<tr>
<td>PDZ2N4</td>
<td>RING-PDZ-PDZ-NLS</td>
<td>568</td>
<td>KRRRRRRR</td>
</tr>
<tr>
<td>RGS2</td>
<td>PDZ-NLS</td>
<td>727</td>
<td>RICKRRR</td>
</tr>
<tr>
<td>RIK51</td>
<td>POZ-C2-NLS-C2</td>
<td>1427</td>
<td>WRRKRRRQFQ</td>
</tr>
<tr>
<td>SHAM6C</td>
<td>ANK-ANK-ANK-ANK-ANK-ANK-ANK-SH3-PDZ-SAM</td>
<td>765</td>
<td>RKXKKDK</td>
</tr>
</tbody>
</table>
| SHAM6C           | ANK-ANK-ANK-ANK-ANK-ANK- | 223 / 932 | KRRRRRRR |}

© The Authors Journal compilation © 2010 Biochemical Society
Figure S3  Sequence and structure of ZO proteins

(A) The first PDZ domains of ZO-1 and ZO-2 are highly similar in sequence and structure. The binding site is highlighted in green and the two non-conserved amino acids in the binding site are displayed in blue (I48V and F42R). These two changes in the binding site are not expected to alter the specificity because position 42 is on the periphery of the binding site and the change at position 48 involves two chemically similar residue types. The binding site was defined as in (1) keeping only amino acids with at least one atom at a distance smaller than 4 Å from the peptide.

(B) Three-dimensional structure of ZO-1 with a bound peptide (WRRTYL) (PDB code 2H2B). The same colour code as in (A) is used to highlight the binding site and the amino acids that are not conserved between ZO-1 and ZO-2.

(C) The ZO-2–PDZm mutant disrupts important binding site positions. The four point mutations of ZO-2–PDZm are displayed in purple.
Figure S4 YAP2 accumulates in the nucleus in the presence of ZO-2-WT in HEK-293 cells

Expression of ZO-2 was induced by tetracycline in HEK-293 cells, and cytosol or nuclear fractions were separated by using the CelLytic™ NuCLEAR™ extraction kit (Sigma–Aldrich), followed by immunoblotting (IB) with indicated antibodies. Molecular masses (MW) are indicated in kDa on the left.

Figure S5 Inhibition of YAP induced transactivation of RUNX2-driven gene expression by ZO-2

HEK-293 cells were transfected with 1 µg of the firefly luciferase reporter construct (p6OSE2-Luc), 50 ng of β-galactosidase, 500 ng of the GFP–YAP construct and increasing concentrations of GFP–ZO-2 DNA (100, 250, 500 and 1000 ng). Total DNA was kept constant using an empty pEGFP vector. At 24 h after transfection, cells were resuspended and seeded in a black 96-well plate (Costar). After another 24 h, luciferase activity from triplicate samples was determined. The cells were lysed in lysis buffer (25 mM Tris/HCl, pH 7.8, 2 mM EDTA, 2 mM DTT (dithiothreitol) 10% (v/v) glycerol and 1% (v/v) Triton X-100) for 15 min at room temperature and 35 µl of luciferase substrate buffer (20 mM tricine, 1.07 mM (MgCO₃)₂Mg(OH)₂·H₂O, 2.67 mM MgSO₄·7H₂O, 0.1 mM EDTA, 33.3 mM DTT, 270 µM coenzyme A, 470 µM luciferin and 530 µM ATP) was added per 50 µl of lysate. Light emission was measured for 5 s in a TopCount chemiluminescence counter (Packard). Luciferase activity, expressed in arbitrary light units, was corrected for protein concentration by normalization to β-galactosidase. Each experimental point was measured in triplicate and the experiment was repeated three times. Results are expressed as means ± S.E.M.
Figure S6  Knockdown of ZO-2 has no influence on the localization of YAP in MDCK cells

(A) Immunostaining of ZO-2 (green) and YAP (red) in control MDCK and MDCK cells treated with siRNA targeting ZO-2. Scale bar, 20 μm. (B) Immunoblot (IB) showing partial knockdown of ZO-2 in MDCK cells.
Figure S7 Conservation of YAP and ZO-2

(A) The YAP C-terminus is well conserved in many species. Amino acids highlighted in blue are not conserved compared with human YAP. (B) The ZO-2 domain architecture is also conserved in most orthologues. GK, GUK. In sea anemone and Hydra, only one ZO protein is present. Note that two potential NLSs (shown in orange) are present in human and chimpanzee ZO-2 proteins (2,3). One of the NLSs is conserved from humans to frog. (C) Multiple sequence alignment of the first PDZ (PDZ1) domains of ZO-2 orthologues. The binding site is shown in green and it is highly conserved for all species in which the YAP C-terminus is conserved. Blue amino acids highlight non-conserved positions compared with the human PDZ1 domain from ZO-2.

REFERENCES


Received 14 June 2010; accepted 24 September 2010
Published as BJ Immediate Publication 24 September 2010, doi:10.1042/BJ20100870

© The Authors. Journal compilation © 2010 Biochemical Society