The Tight Junction Protein ZO-2 Localizes to the Nucleus and Interacts with the Heterogeneous Nuclear Ribonucleoprotein Scaffold Attachment Factor-B*

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Andreas Traugger‡, Renate Fuchs‡, Istvan A. Krizbaiš, Thomas M. Weiger®, Hans-Christian Bauer‡, and Hannelore Bauer‡

From the "Institute of Molecular Biology, Austrian Academy of Sciences, 5020 Salzburg, Austria, the "Institute of Biophysics, Biological Research Center, 6723 Szeged, Hungary, and the "Institute of Zoology, University of Salzburg, 5020 Salzburg, Austria

Zonula occludens proteins (ZOPs), currently comprising ZO-1, ZO-2, and ZO-3, belong to the family of membrane-associated guanylate kinase homologue (MAGUK) proteins that are involved in the organization of epithelial and endothelial intercellular junctions. ZOPs bind to the cytoplasmic C termini of junctional transmembrane proteins linking them to the actin cytoskeleton. They are characterized by several conserved modules, including three PDZ domains, an SH3 domain, and a guanylate kinase-like domain, elements indicating that ZOPs may serve multiple purposes. Interestingly, ZOPs contain some unique motifs not shared by other MAGUK family members, including nuclear localization and nuclear export signals and a leucine zipper-like sequence. Their potential involvement in cell growth and proliferation has been suggested earlier based on the observation that the N-terminal half of ZOPs displays significant similarity to the product of the Drosophila tumor suppressor gene lethal(1)disclarge (dlg). The nuclear targeting of ZOPs in subconfluent epithelial cell cultures is well documented, although the action of the junctional MAGUKs in the nucleus has remained elusive. Here we show for the first time that nuclear ZO-2 directly interacts with the DNA-binding protein scaffold attachment factor-B (SAF-B). Our results from two-hybrid assays and in vivo co-immunoprecipitation studies provide evidence to suggest that ZO-2 associates with the C-terminal portion of SAF-B via its PDZ-1 domain. We further demonstrate that enhanced green fluorescent protein (EGFP)- and DsRed-tagged ZO-2 and SAF-B fusion proteins partially co-localize in nuclei of transfected epithelial cells. As shown by laser confocal microscopy and epifluorescent analysis, nuclear ZO-2 is present in epithelial and endothelial cells, particularly in response to environmental stress conditions. Interestingly, no association of SAF-B with ZO-1 was found, which supports the notion that junctional MAGUKs serve nonredundant functions.

Tight intercellular contacts (tight junctions (TJs))1 are responsible for the “barrier” function of epithelia and endothelia restricting the paracellular transport of solutes and molecules across epithelial and endothelial cell sheets (1–5). TJs consist of several transmembrane components that interact with a network of “peripheral” cytoplasmic proteins. So far, three types of TJ-related transmembrane proteins: occludin, claudins, and junctional adhesion molecule, have been described, all of them associating with at least one of the Zonula occludens proteins (ZOPs) (for review see Refs. 6–8). ZOPs, in turn, establish a link between the junction site and the cytoskeleton by interacting directly with actin filaments (9–11). The observation that ZOPs not only associate with each other but also with components of adherens junctions and gap junctions in cells lacking TJs (12–14) suggests a universal role for ZOPs at the cytoplasmic surface of the junctional plaque.

ZOPs are structurally similar to the membrane-associated guanylate kinase homologue (MAGUK) family of signaling molecules (for review see Refs. 15 and 16). These multidomain proteins contain a core of specific modules composed of three PDZ domains, an SH3 domain, a terminal acidic domain, and an enzymatically inactive guanylate kinase-like domain. Apart from theses classical MAGUK domains, ZOPs contain some unique motifs not shared by other MAGUK family members, including nuclear localization and nuclear export signals and a leucine zipper (16).

The first MAGUK identified was the product of the Drosophila tumor suppressor gene lethal(1)disclarge (dlg), defining the subfamily of dlg-like MAGUKs, which also includes the mammalian synaptic density protein PSD-95/SAP90. The dlg gene was actually identified by the tumorous overgrowth seen in the imaginal discs of larvae carrying loss-of-function mutations (17, 18). Mammalian ZOPs, comprising ZO-1, ZO-2, and ZO-3, and the Drosophila Tamou, constitute the ZO-1-like MAGUK subfamily. Mammalian members of this subfamily localize to TJs, whereas the Drosophila member localizes at septate junctions. ZO-1 (220 kDa) was the first protein suggested to be associated with TJs of epithelial and endothelial cells (19, 20). ZO-1 is particularly enriched at the TJs of epithelial and endothelial cells but has also been found in astrocytes, fibroblasts, sarcoma, and myeloma cell lines (for review see Ref. 16). ZO-2, a 160-kDa phosphoprotein is highly homologous to ZO-1 and was

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2 To whom correspondence should be addressed. E-mail: hcbauer@imb.oeaw.ac.at.
3 The abbreviations used are: TJ, tight junction; ZOP, Z. occludens protein; MAGUK, membrane-associated guanylate kinase homologue; SAF-B, scaffold attachment factor-B; NLS, nuclear localization signal; NES, nuclear export signal; aa, amino acids; MDCK, Madin-Darby canine kidney; BSA, bovine serum albumin; PVDF, polyvinylidene difluoride; Pipes, 1,4-piperazinediethanesulfonic acid, EGFP, enhanced green fluorescent protein.

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found to co-precipitate with ZO-1 in epithelial cells (14, 21). ZO-2 has a similar domain structure to ZO-1, but the two proteins diverge at the C terminus, where ZO-2 contains an alternatively spliced region called the β-motif (22). Finally, the 130-kDa protein ZO-3, originally described as p130, has been added to the list. ZO-3 shows high homology to ZO-1 and ZO-2 but contains a unique proline-rich region located between the second and third PDZ domain (23). So far, no alternatively spliced domain has been detected in ZO-3. Further, ZO-3 directly interacts with ZO-1 and the cytoplasmic domain of occludin, but not with ZO-2. The divergence in the C termini of ZO-1 like MAGUKs suggests that these proteins may have significant functional differences. Furthermore, ZOPs not only interact with occludin, claudins, and junctional adhesion molecule but also associate with a variety of cytoplasmic proteins of less defined function, including the myosin-binding protein cingulin (24), the Ras effector AF-6 (25), and the erythrocYTE actin-binding protein 4.1 (26).

In recent years observations have accumulated indicating that ZO-2 localizes to the nucleus in epithelial cells (27, 28). Up to now, the biological relevance of this nuclear shuttling has remained elusive.

We now report that nuclear ZO-2 interacts directly with the DNA-binding protein scaffold attachment factor-B (SAF-B) (29). This interaction has been identified and confirmed by use of a yeast-based two-hybrid screen and in vitro co-immunoprecipitation studies. ZO-2 appeared to interact specifically with SAF-B but not with ZO-1. Using epitope-tagged ZO-2 fusion constructs, we have tested for the functional significance of the predicted nuclear localization and export signals (NLS and NES). Here we demonstrate that more than two NESs are active in driving the nuclear export of ZO-2. Also the previously proposed NLS does not seem to be the only targeting information domain responsible for the nuclear localization of ZO-2. We further show that EGF/DEd2-tagged fusion proteins of ZO-2 and SAF-B substantially co-localize in nuclei of MDCK cells and that increased nuclear staining of ZO-2 is observed in epithelial cells subjected to environmental stress conditions.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pGBKTT7–5′–ZO-2 was generated by subcloning the coding sequence of the N-terminal region of ZO-2 (aa 1-470; GenBank™ accession number AF113005) into the yeast two-hybrid vector pGBKTT7 (Clontech). pGBKTT7–PDZ-1 encodes aa 18–99, and pGBKTT7–PDZ-2 encodes aa 301–369 of ZO-2 (GenBank™ accession number L27152). pEGF-SAF-B encodes aa 1–874, pEGF/SAF-B and pDsRed2/SAF-B encodes 425–826, and pGADT7/C-term.SAF-B encodes aa 445–826 of rat SAF-B (GenBank™ accession number AP065324).

The following ZO-2 constructs (according to GenBank™ accession number AAC373329) were generated: pEGFP-ZO-2 (aa 1–1174); pEGFP-5′–ZO-2 (aa 1–470); pEGFP-5′–ZO-2–N1 (aa 1–358); pEGFP-5′–ZO-2–N1N2–ES1 (aa 358–653); pEGFP-5′–ZO-2–N1N2–ES1 (aa 358–653); pEGFP-ZO-2-mut (aa 1–1174; NES1 located at aa 361–369 mutated to LVEQVLRDS to LQVQVRSDD); and pEGFP-ZO-2–2xmut (aa 1–738; NES1 mutated as above, NES2 located at aa 728–738 mutated to LELKANEPLDL to LELKANEFPDE).

Antibodies—Monoclonal antibodies directed against c-Myc (Santa Cruz), GFP (Clontech), and β-tubulin (Sigma) were obtained from commercial sources. Polyclonal anti-ZO-2, anti-occludin, and anti-claudin-1 antibody was purchased from Zymed Laboratories Inc., rabbit anti-integrin αv antibody was obtained from Chemicon, and polyclonal anti-ubiquitin was from Santa Cruz. Antibodies directed against human SAF-B were kindly provided by Dr. Frank O. Fackelmann (Heinrich Pette-Institute, University of Hamburg, Hamburg, Germany). Monoclonal anti-SAF-B antibody was purchased from Abcam. Alexa Fluor™ 568 goat anti-rabbit IgG was obtained from Molecular Probes. Preimmune serum was purchased from Pierce (ImmunoPure® normal rabbit serum).

Cell Culture, DNA Transfections, and Indirect Immunofluorescence—LLC-PK1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. MDCK cells were cultured in minimum essential medium enriched with 10% fetal calf serum. For transfection experiments the cells were grown to 70–80% confluency and transfected in suspension with Effectene™ transfection reagent (Qiagen) according to the manufacturer’s protocol.

Yeast Two-hybrid Screening and β-Galactosidase Assays—Two-Hybrid screening experiments were performed essentially as described (30). Briefly, the Saccharomyces cerevisiae reporter strain AH109 expressing the N-terminal region of ZO-2 (pGBKTT7–5′–ZO-2) was transformed with a mouse liver MATCHMAKER™ cDNA library (Clontech). The transformants were then plated onto appropriate selective medium (SD/−Trp/−His/−Leu/+ Trp). All of the positive clones were further processed as described and assayed for β-galactosidase activity by a colony lift filter assay as recommended by the manufacturer (Clontech). Further, the candidate clones were re-streaked onto SD/−His/−Leu/+ Trp + 100 μg/ml 3-aminoazoni toazo to confirm the interaction.

Preparation of Nuclear Extracts—The nuclei were isolated according to the sucrose/citric acid method (31). MDCK cells were harvested by trypsinization and washed in phosphate-buffered saline. If the cells were treated with 60 μM CdCl₂ or grown at 42 °C (for 2 h), the cell cultures contained 5 μM His₃-Leu₄-TrpCl, 5 μM 3-aminotriazole. The cells were harvested, washed, and resuspended in buffer A (10 mM Tris·Cl, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride), and after an incubation on ice for 5 min, the cells were centrifuged at 500 × g at 4 °C. The supernatant was discarded, and the pellet was resuspended in buffer B (10 mM Tris·Cl, pH 7.2, 0.1 mM phenylmethylsulfonyl fluoride). The cells were homogenized in a tight fitting glass/Teflon potter on ice (40 strokes at 800 rpm) followed by centrifugation at 700 × g for 5 min (4 °C). The collected nuclei were resuspended in ice-cold solution of 0.88 μM sucrose, 1.5% citric acid and were given another five strokes at 800 rpm. The homogenate was carefully under-layered with ice-cold solution of 2.5 μM sucrose, 1.5% citric acid and centrifuged at 1000 × g for 10 min (4 °C). The layer containing the nuclei was carefully aspirated and washed with a large volume of buffer C (10 mM HEPES, pH 7.5, 120 mM KCl, 10 mM NaCl, 2 mM CaCl₂, 1.1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A). The isolated nuclei were centrifuged as indicated above and were either resuspended in SDS sample buffer or further processed for scanning electron microscopy according to standard procedures.

Far Western Analysis—Far Western analysis was performed as described previously (32). Briefly, the proteins were extracted from MDCK cells by incubation in cell lysis buffer (20 mM Tris·Cl, pH 7.4, 150 mM NaCl, 5 mM Na₂VO₄, 10 mM NaF, 1% Triton X-100, 0.1% SDS, 1 mM Pefabloc SC, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A) for 15 min on ice. Cell debris was collected by centrifugation at 14,000 × g for 15 min at 4 °C. Supernatant was transferred into micro tubes (2 μg of anti-ZO-1, anti-ZO-2, anti-occludin, or rabbit preimmune serum were added to the samples, and the immunoprecipitations were carried out as described below.

The samples and BSA (50 μg/ml as negative control) were separated by SDS-PAGE and were transferred to PVDF membranes (Pall Life Sciences). The membranes were blocked with TBS-T (20 mM HEPES, pH 7.5, 25 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, 5% skimmed milk powder) at room temperature followed by an incubation in Hyb 75 (20 mM HEPES, pH 7.5, 75 mM KCl, 2.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05% Nonidet P-40, 1% skimmed milk powder) for 15 min at room temperature.

35S-labeled SAF-B and ZO-2 were synthesized in vitro using TNT™ T7 Coupled Reticulocyte Lysate System (Promega). The probes were diluted in 3 ml of Hyb 75, and the membranes were incubated for 5 h at 4 °C. Finally, the membranes were washed three times in Hyb 75 for a total of 45 min, dried, and exposed to an x-ray film (Kodak).
Interaction of ZO-2 with SAF-B

In Vitro Binding Assays, Co-immunoprecipitation, and Immunoblotting—

***T*-Labeled, c-Myc-tagged fragments of ZO-2 (PDZ-1 and PDZ-2) and SAF-B (C-term.SAF-B) were synthesized using the TNT™ T7-coupled reticulocyte lysate system (Promega). Equal amounts of protein were combined and incubated at 30 °C for 1.5 h followed by the addition of 500 μl of co-immunoprecipitation buffer (0.2% Triton X-100, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 5 mM Na₂VO₄, 10 mM NaF, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 5 μg/ml Pefabloc SC, 10% glycerol) and 2 μg of mouse monoclonal anti-c-Myc. The samples were rotated for 2 h at 4 °C followed by the addition of 20 μl of 1:1 slurry of protein G-Sepharose 4 Fast Flow (Amersham Biosciences). The immunocomplexes were then collected for 3 h at 4 °C and washed four times with 1 ml of co-immunoprecipitation buffer. The bound proteins were separated by SDS-PAGE. After electrophoresis the gels were fixed in 30% methanol, 10% acetic acid, soaked for 30 min in Amplify™ (Amersham Biosciences), and dried under vacuum at 80 °C. The dried gels were then exposed to x-ray films (Kodak).

For co-immunoprecipitation experiments LLC-PK1 cells were transfected with pEGFP-SSAF-B and were lysed in lysis buffer (20 mM Pipes, pH 6.1, 83 mM KCl, 17 mM NaCl, 1 mM MgCl₂, 5 mM EDTA, 1 mM dithiothreitol, 10 mM NaF, 5 mM Na₂VO₄, 0.5% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μM Pefabloc SC). After clearing the lysate by centrifugation (14,000 rpm; 15 min; 4 °C), co-immunoprecipitations and immunoblotting were performed as described by us earlier (30). For co-immunoprecipitation of endogenous SAF-B with endogenous ZO-2, 293E cells were incubated on ice for 2 h in X-link buffer (phosphate-buffered saline, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 10 mM NaF, 5 mM Na₂VO₄, 0.1% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μM Pefabloc SC). The cross-linking reaction was stopped by adding 1 μl Tris-Cl (pH 7.5) to 10 ml, Nonidet P-40 to 1%, and SDS to 0.05% final concentration, respectively. The cells were lysed on ice for 30 min, and the lysate was centrifuged at 16,000 × g for 12 min. The supernatant was split into three tubes, and 1.5 μg of anti-ZO-2 or anti-ubiquitin or no antibody was added to the samples followed by an incubation for 2 h at 4 °C. The immunocomplexes were collected overnight (4 °C) by incubating the samples with 20 μl of protein A/G-Sepharose 4 Fast Flow (Amersham Biosciences). The beads were recovered by centrifu- gation at 1,600 × g for 10 min, and the lysate was washed four times with 1 ml of wash buffer (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM CaCl₂, 10 mM NaF, 5 mM Na₂VO₄, 0.1% Nonidet P-40, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μM Pefabloc SC). The bound proteins were analyzed by SDS-PAGE and immunoblotting.

For quantitative Western blot, the protein content was determined using a BCA™-200 protein assay kit (Pierce). Of each sample 10 μg of protein were loaded and processed for immunoblotting as described earlier. Three independent experiments were performed, and three densitometric measurements/experiment were carried out using the Gel-Pro Analyzer 3.1 software from Media Cybernetics.

RESULTS AND DISCUSSION

We have observed previously that ZO-2 localizes to the nucleus of highly migratory endothelial cells (33). To elaborate our earlier findings we have set out to identify novel interaction partners of ZO-2 that might elucidate the biological significance of its nuclear targeting.

The results from our two-hybrid studies are shown in Fig. 1. We have used the N-terminal domain of ZO-2 (pGBKTT7-5’-ZO-2), comprising PDZ-1 and PDZ-2, as a bait to screen a MATCHMAKER™ mouse liver cDNA expression library. One putative positive clone (AT1) was chosen and further tested by a β-ga-
lactosidase assay and by restreaking onto a SD−His−Leu−Trp + 100 mM 3-aminotriazole selection agar plate. As shown in Fig. 1A, only AT1 (3) and the positive control (pGBK7-p53/pGADT7-T) showed 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) staining and were able to grow on the selection agar plate. The insert of AT1 was isolated, sequenced, and

**Fig. 2. Isolation of MDCK cells nuclei and interaction of ZO-2 with SAF-B in the nucleus.** A, scanning electron micrograph from isolated nuclei. The higher magnification (lower panel) demonstrates the intact nuclear membrane. B, marker proteins monitored for nuclear fraction purity. Equal amounts of separated protein were immobilized on a PVDF membrane and were immunoprobe for ZO-2, SAF-B, β-tubulin, and integrin α3. N, nuclear extract; T, total cell extract. Whereas high amounts of the nuclear protein SAF-B could be detected in the nuclear extract, only minimal amounts of the cytoplasmic protein β-tubulin and the plasma membrane protein integrin α3 could be detected, reflecting the purity of the nuclei preparation. Further, ZO-2 was detected in both the total cell and the nuclear fraction of MDCK cells. C, ZO-2 was immunoprecipitated from nuclear extracts, separated by SDS-PAGE, and transferred to a PVDF membrane. As a negative control, rabbit preimmune serum (50 μg) was added to the nuclear extract for immunoprecipitation (lanes 2 and 6). The immobilized proteins were then probed with 35S-labeled SAF-B protein. SAF-B specifically interacts with nuclear ZO-2 (lane 1, arrowhead), but not with BSA (50 μg loaded; lane 3) or the membrane (lane 4, empty lane). Aliquots of the samples where immunoprecipitated with an ZO-2 antibody. Lane 5, ZO-2 IP; Lane 6, preimmune serum IP. D, MDCK cells co-transfected with pEGFP-5′-ZO-2-ΔNES1 and pDsRed2-fSAF-B were fixed in 100% methanol and treated with 4′,6-diamidino-2-phenylindole (DAPI) to stain DNA (blue). 5′-ZO-2-ΔNES1-EGFP (green) specifically localizes to the nucleus in a speckled pattern and partially co-localizes with DsRed2-fSAF-B (red). IB, immunoblot.
subjected to a BLAST2 sequence homology search. At the nucleotide and protein level, AT1 revealed the highest homology (95% identity) to SAF-B (GenBank™ accession number AF056324). The isolated AT1 cDNA corresponds to nucleotides 1539–2747 (aa 425–826) of rat SAF-B (total length, 874 aa) and thus is referred to as ISAF-B below.

To substantiate the interaction of SAF-B with ZO-2, in vitro co-immunoprecipitation experiments were performed. LLC-PK1 cells were transiently transfected with pEGFP-3SAF-B, lysed, and subjected to immunoprecipitation with a polyclonal antibodies. The resulting blots were immunoprobed with α-ZO-2 antibody. For control studies immunoprecipitations were performed with preimmune serum or without antibody. The resulting blots were immunoprobed with α-GFP antibodies. As shown in Fig. 1B, the EGFP-tagged 3SAF-B efficiently co-precipitated with endogenous ZO-2 (Fig. 1B, lane 1), whereas no unspecific interactions were observed (Fig. 1B, lanes 2 and 3).

Finally, endogenous SAF-B interacts with endogenous ZO-2 immunoprecipitated from 293 E cells (Fig. 1C, lane 1). However, SAF-B only co-immunoprecipitated with ZO-2 efficiently if the cells were lysed in the presence of the chemical cross-linker DSP, suggesting that the association between ZO-2 and SAF-B is unstable in lysed cells. Nevertheless, a weak interaction was also observed in the absence of DSP (data not shown).

The direct and specific association of endogenous ZO-2 with SAF-B was further evidenced by far Western blot analysis. MDCK cells were lysed, and immunoprecipitations were performed by the addition of antibodies against ZO-1, ZO-2, and occludin or preimmune serum (control). The resulting samples were separated by SDS-PAGE, blotted, and hybridized with 35S-labeled SAF-B or lamin C (control). Fig. 1D clearly demonstrates that SAF-B exclusively interacts with ZO-2 (lane 2), but neither with ZO-1 nor with occludin (lanes 1 and 3). Further, no unspecific binding was found with the unrelated probe lamin C (not shown).

Next we determined which of the PDZ domains present in the original “bait” (5’-ZO-2) interacts with SAF-B. 35S-Labeled Myc-tagged fragments of ZO-2 comprising either PDZ-D1 or PDZ-D2 were synthesized in vitro and incubated with 35S-labeled C-term.SAF-B. The samples were precipitated with a monoclonal α-c-Myc antibody, electrophoresed, and analyzed by fluorography. Fig. 1E demonstrates that only PDZ-D1 (lane 1) interacts with the C-terminal portion of SAF-B (C-term.SAF-B). Only slight unspecific binding was observed with PDZ-D2 and lamin C (Fig. 1E, lanes 2 and 3).

Using Western blot analysis we have further shown that ZO-2 is present in the nuclear fraction derived from MDCK cells (Fig. 2). As expected, the major portion of ZO-2 was found in total cell extracts, but a substantial amount was also detectable in the nuclear fraction (Fig. 2B). The purity of the nuclear pellet was monitored by electron microscopy (Fig. 2A) and by marker protein staining (Fig. 2B). SAF-B (nuclear marker) was exclusively present in the nuclear fraction, whereas β-tubulin (cytoplasmic marker) and integrin αv (plasma membrane marker) were predominantly traceable in whole cell extracts (Fig. 2B). Further, the electron micrograph showed that nuclear membranes were still intact after the nuclei isolation procedure (Fig. 2A).

We further demonstrate that nuclear ZO-2 is capable of interacting with 35S-labeled SAF-B (Fig. 2C, upper panel, arrowhead). For this experiment endogenous ZO-2 was immunoprecipitated from nuclear extracts using an α-ZO-2 antibody. As a negative control, rabbit preimmune serum was used for immunoprecipitation. The samples were then electrophoresed, blotted, and hybridized with 35S-labeled SAF-B or probed with an α-ZO-2 antibody. As a result, SAF-B specifically interacted with nuclear ZO-2 (Fig. 2C, lane 1, arrowhead) but not with proteins precipitated by the preimmune serum (lane 2). No unspecific binding to BSA or to the PVDF membrane was observed (lanes 3 and 4). The lower panel of Fig. 2C demonstrates the specific immunoprecipitation of ZO-2 from nuclear extracts (lane 5). No ZO-2 could be detected in the negative control using preimmune serum for immunoprecipitation (lane 6).

The co-localization of SAF-B and ZO-2 in the nucleus of MDCK cells was monitored using EGFP/DsRed2-tagged proteins. Fig. 2D shows considerable overlaps of the nuclear speckles corresponding to 5’-ZO-2-ΔNES1-EGFP and DsRed2-3SAF-B. Together, these results strongly support the suggestion that the interaction of ZO-2 with SAF-B takes place in the nucleus.

It was suggested previously that ZO-2 contains two putative nuclear export signals (NES1 and NES2) and one prominent NLS (28). This is consistent with results from our sequence analyses using various prediction programs. To determine whether these leucine-rich motifs indeed are involved in the nuclear targeting of ZO-2, we have performed localization studies using EGFP-tagged ZO-2 mutants. Various ZO-2 constructs lacking one or more of the predicted domains were generated: (i) 5’-ZO-2, carrying NES 1 and the predicted NLS, (ii) 5’-ZO-2-ΔNES1, lacking NES 1 but carrying the NLS, (iii) 5’-ZO-2-ΔNES1ΔNLS, lacking both the NLS and NES1 motif, (iv) ZO-2 mut containing a mutation only in NES1, and (v) ZO-2 2xmut carrying mutations in both NES domains. Mutated proteins were expressed as EGFP fusion products in MDCK cells. Our results from transfection studies are shown in Fig. 3B. Although 5’-ZO-2 does not accumulate in the nucleus (Fig. 3B, panel I), 5’-ZO-2-ΔNES1 perfectly localizes to the nucleus (Fig. 3B, panel II), suggesting that NES 1 is a functional domain and is sufficient to drive the nuclear export of the N-terminal portion of ZO-2. Deletion of the NLS motif (5’-ZO-2-ΔNES1ΔNLS) did not abolish the nuclear targeting of this ZO-2 mutant (Fig. 3B, panel III), which indicates that one or more additional NLS domain(s) must be present in the N-terminal region of ZO-2. Interestingly, 5’-ZO-2-ΔNES1ΔNLS was uniformly distributed in the nucleus, and hardly any speckles were visible. This may be explained by the fact that this construct lacks the PDZ-1 domain and thus is not capable of interacting or co-localizing with SAF-B. The fusion proteins ZO-2 mut (Fig. 3B, panel IV) and ZO-2 2xmut (Fig. 3B, panel V) were found to be exported from the nucleus, suggesting that yet unidentified NES domain(s) situated between NES1 and NES2 must be active in ZO-2.

The nuclear presence of a junctional MAGUK was first demonstrated by Gottardi et al. (34), showing that before cell maturation and during remodeling of cell-cell contacts, particular ZO-1 staining is visible in cultured MDCK cells. Complementing these results González-Mariscal et al. (27) and Islas et al. (28) reported recently the localization of ZO-2 in the nucleus of subconfluent epithelial cell monolayers. Here we have shown that ZO-2 localizes to the nucleus of endothelial and epithelial cells although to varying extents (Fig. 4A). Highly migratory endothelial cells exhibit considerable nuclear ZO-2 staining with a distinct speckled pattern (Fig. 4A). In the nuclei of epithelial cells, ZO-2 immunoreactivity is low (not shown) but increases markedly in response to environmental stresses such as heat shock (Fig. 4, A and B) and chemical insult (Fig. 4B). Interestingly, ZO-2 staining is visible in the perinuclear area of all cell types inspected. Under physiological stress conditions, ZO-2 seems to be recruited to the nucleus, whereas cytoplasmic ZO-2 immunoreactivity diminishes and the localization of ZO-2 at plasma membranes appears unchanged. Fig. 4B demon-
strates the relocalization of nuclear and perinuclear ZO-2 in epithelial cells following chemical stress (CdCl₂) and heat shock (42 °C). Quantitative Western blot experiments demonstrate that nuclear ZO-2 levels increase upon physiological stress conditions, especially in response to CdCl₂ treatment (Fig. 4C, right panel). The purity of the nuclear fraction was monitored as described above. Interestingly, the overall amount of ZO-2 protein is not significantly changed (Fig. 4C, left panel).

To test whether the nuclear accumulation of 5’-ZO-2, found to interact with SAF-B, influences other TJ-associated proteins, LLC-PK1 cells were transiently transfected with pEGFP-5’-ZO-2-ΔNES1 and stained for occludin, claudin-1, and ZO-1. As shown in Fig. 5, the localization of other TJ-related proteins appeared unchanged in cells overexpressing the nuclear ZO-2 fusion protein.

Originally, SAF-B has been described as one of the abundant nuclear proteins that function in chromatin structure by interacting with scaffold or matrix attachment DNA elements (29). In many cases, these elements co-map with boundaries of actively transcribed domains and have therefore been considered to exert regulatory effects on adjacent genes. Although SAF-B does not exhibit any homology to other previously identified DNA-binding proteins, it nevertheless shares the ability for nucleic acid dependent self-aggregation with other scaffold or matrix attachment DNA-binding proteins such as histone H1 (35) and topoisomerase II (36). SAF-B was shown to be highly concentrated in nuclear speckles co-localizing with SC-35 and to directly interact with various splicing factors and RNA polymerase II via its C-terminal domain (37). Sequence analysis revealed that SAF-B is identical to the nuclear protein HAP (hnRNP A1-associated protein) (38) and to HET (Hsp27-ERE-TATA-binding protein), which was found to exert a repressive action on the HSP27 promoter in human breast cancer cells (39).

During the last couple of years, intriguing evidence has accumulated suggesting that junctional MAGUKs not only serve as structural “organizers” at the plasma membrane linking the junctional plaque to the cytoskeleton but are also potentially involved in cell growth and transcriptional regulation. Convincing studies have been performed with Drosophila, showing that junctional proteins functioning in epithelial differentiation simultaneously act as tumor suppressors and are thus involved in growth control. As mentioned before, the N-terminal domain of ZOPs is homologous to the Drosophila tumor suppressor protein discs-large A (DlgA), a component of invertebrate septate junctions (40). Dlg proteins with mutations in the PDZ and SH3 domains were found to cause neoplastic overgrowth of larval imaginal disc epithelial cells (17). Comparably, another invertebrate orthologue of the mammalian ZOPs, the Drosophila tamou gene, has been implicated in regulating the expression of the tumor suppressor helix-loop-helix protein extramacrochaetae, leading to the overgrowth of proneural cells in the fly (41). Mutants of ZO-1, which encode the PDZ domains but no longer localize at the plasma membrane, were shown to induce a fibroblastoid, transformed phenotype of MDCK cells in vitro, and an increased tumorigenicity
FIG. 4. Laser confocal and epifluorescent analysis of nuclear ZO-2 in endothelial and epithelial cells. A, highly migratory endothelial cells (cECs) and heat-shocked epithelial LLC-PK1 cells were fixed and then processed for laser confocal microscopy using a polyclonal α-ZO-2 antibody. B, LLC-PK1 cells were incubated for 2 h at the presence of 60 μM CdCl₂ or were grown at 42 °C for 2 h. The cells were then methanol-fixed and stained with a rabbit α-ZO-2 antibody. As a control, untreated cells were analyzed. C, nuclei from untreated (lane 1), CdCl₂-treated (lane 2), and heat-shocked (lane 3) MDCK cells were isolated and equal amounts of nuclear extracts (10 μg/sample) were run on each lane (upper right panel). Further, total cell extracts from stressed and untreated cells were prepared and 10 μg of protein were loaded per lane (upper left panel). A representative example of a ZO-2 Western blot is shown (top panels) in combination with the corresponding densitometric measurement of ZO-2 levels (bottom panels). The data represent the means ± S.D. of three independent experiments. Further, three densitometric measurements/experiment were performed. I.O.D., integrated optical density (arbitrary units).
of these cells in vivo (42). A detailed study concerning the expression of ZO-2 in normal and neoplastic human tissue has been reported by Chlenski et al. (43). The authors have shown that ZO-2 is rarely abnormal in colon and prostate cancer but was de-regulated in most of the breast cancer samples. This indicates that activation or inactivation of ZO-2 expression may require participation of tissue-specific transcription factors. Last but not least, the recent report about the interaction of ZO-1 with the Y-box transcription factor ZONAB (ZO-1 associated nucleic acid binding protein) has further emphasized the notion of a potential “dual” role of ZOPs (44).

We report here that the tight junction-associated protein ZO-2 interacts directly with the DNA-binding protein SAF-B (19). This will further elucidate the role of junctional MAGUKs in a signaling cascade linking TJs to cell growth and proliferation.

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The Tight Junction Protein ZO-2 Localizes to the Nucleus and Interacts with the Heterogeneous Nuclear Ribonucleoprotein Scaffold Attachment Factor-B
Andreas Traweger, Renate Fuchs, Istvan A. Krizbai, Thomas M. Weiger, Hans-Christian Bauer and Hannelore Bauer

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