ARTICLE
Journal of Cellular Biochemistry 113:934–945 (2012)

Journal of Cellular Biochemistry

The Hinge Region of the Scaffolding Protein of Cell Contacts, Zonula Occludens Protein 1, Regulates Interacting With Various Signaling Proteins

Manjot Singh Bal,[1] Victor Castro,[1] Jörg Piontek,[1] Christine Rueckert,[1] Juliane K. Walter,[1] Aliaksei Shymanets,[2] Barbara Kurig,[2] Hannelore Haase,[3] Bernd Nürnberg,[2] and Ingolf E. Blasig[1*]

[1] *Leibniz-Institut für Molekulare Pharmakologie, Berlin-Buch, Germany*

[2] *Department of Pharmacology and Experimental Therapy, Institute of Experimental and Clinical Pharmacology and Toxicology, Eberhard-Karls-University Hospitals and Clinics, and Interfaculty Center of Pharmacogenomics and Drug Research (ICePhA), University of Tübingen, Tübingen, Germany*

[3] *Max-Delbrück-Center for Molecular Medicine, Berlin-Buch, Germany*

ABSTRACT

Zonula occludens protein 1 (ZO-1) is a ubiquitous scaffolding protein, but it is unknown why it functions in very different cellular contacts. We hypothesized that a specific segment, the unique hinge region, can be bound by very different regulatory proteins. Using surface plasmon resonance spectroscopy and binding assays to peptide libraries, we show, for the first time, that the hinge region directly interacts with disparate signal elements such as G-proteins alpha 12 and alpha i2, the regulator of G-protein signaling 5, multifunctional signaling protein ahnak1, and L-type Ca^{2+}-channel beta-2-subunit. The novel binding proteins specifically bound to a coiled coil-helix predicted in the hinge region of ZO-1. The interactions were modulated by phosphorylation in the hinge helix. Activation of the G-proteins influenced their association to ZO-1. In colon cells, G alpha i2 and ZO-1 were associated, as shown by coimmunoprecipitation. After cotransfection in kidney cells, G alpha i2 barely colocalized with ZO-1; the colocalization coefficient was significantly increased when epinephrine activated G-protein signaling. In conclusion, proteins with different regulatory potential adhere to and influence cellular functions of ZO-proteins, and the interactions can be modulated via its hinge region and/or the binding proteins. J. Cell. Biochem. 113: 934–945, 2012.

KEY WORDS: CELL–CELL CONTACTS; PROTEIN–PROTEIN INTERACTION; REGULATORY PROTEINS; SURFACE PLASMON RESONANCE SPECTROSCOPY; PEPTIDE BINDING ASSAYS; G-PROTEINS

Epithelia and endothelia are specialized tissue layers that line the outer and inner surfaces of the body and organs, effectively separating but simultaneously regulating the homeostasis of the two biological compartments they separate. The epithelial cells and endothelial cells, respectively, are bound to each other by cell–cell contacts, such as adherens junctions, gap junctions, or tight junctions (TJ). The TJ seal and regulate the tightness of the intercellular space and they are crucial for the maintenance and regulation of the epithelial/endothelial barrier function.

Zonula occludens protein 1 (ZO-1) is a cytosolic membrane-associated guanylate kinase homologue protein (MAGuK), scaffolding very different types of cell–cell contacts. It does not only anchor transmembranal junction proteins and links them to the cytoskeleton, but also binds a number of other molecules, effectively associating them with the junctions (scheme in Table I). ZO-1 contains protein-binding domains, for example, PDZ, SH3, or GuK. It recruits TJ proteins, such as the paracellularly sealing or pore forming claudins via PDZ-1 and -2 [Itoh et al., 1999] as well as junctional adhesion molecules (JAM) via PDZ-2 or -3 [Bazzoni et al., 2000]. PDZ-2 leads to dimerization of ZO-1 [Utepbergenov et al., 2006] and interacts with the gap junction components connexins [Chen et al., 2008]. Integrins within desmosomes are also

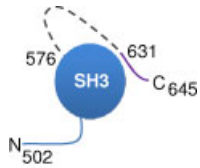
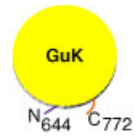
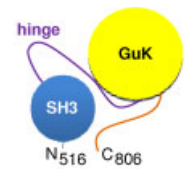
Manjot Singh Bal and Victor Castro contributed equally to this work.

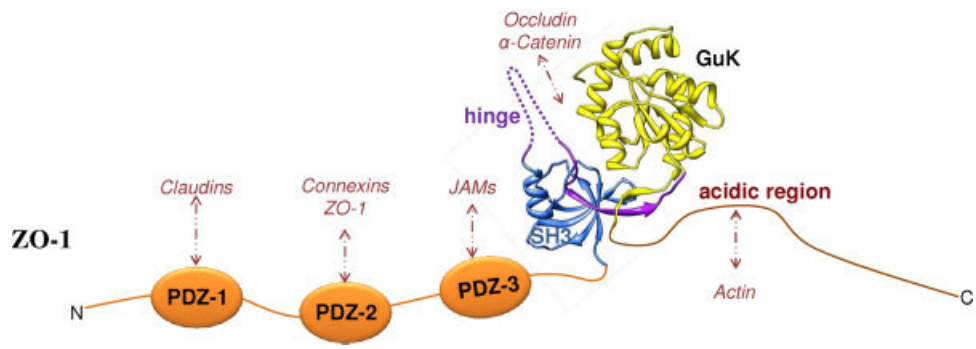
Grant sponsor: DFG; Grant numbers: BL308/6-4, 7-4, 9-1.

*Correspondence to: Ingolf E. Blasig, Leibniz-Institut für Molekulare Pharmakologie Robert-Rössle-Str. 10, 13125 Berlin, Germany. E-mail: iblasig@fmp-berlin.de

Received 3 June 2011; Accepted 14 October 2011 • DOI 10.1002/jcb.23422 • © 2011 Wiley Periodicals, Inc.
Published online 22 December 2011 in Wiley Online Library (wileyonlinelibrary.com).

TABLE I. Demonstration of Direct Association Between the SH3-Hinge-GuK Unit of ZO-1 and Potentially Regulatory Proteins Compared to the Control (MBP), Measured by Surface Plasmon Resonance Spectroscopy

	Bound amount of superinfused ZO-1 construct (ΔRU/mol × 4 min)		
			
Immobilized protein	**SH3-domain**	**GuK-domain**	**SH3-hinge-GuK unit**
Occludin	7.34 ± 1.36	10.00 ± 1.14	18.92 ± 6.52[b]
$G\alpha_{12}$	0.15 ± 0.03	3.71 ± 0.04	8.46 ± 0.10[c]
$G\alpha_{i2}$	0.38 ± 0.07	6.31 ± 0.16	13.26 ± 0.04[d]
RGS5	2.72 ± 0.12	2.16 ± 0.26	4.57 ± 0.44
Ahnak1	−0.52 ± 0.01	1.58 ± 0.05	4.04 ± 0.07
β2-subunit	−2.74 ± 0.02[a]	9.82 ± 0.18[a]	6.96 ± 0.46[a]
MBP	0.55 ± 0.16	0.27 ± 0.22	0.61 ± 0.20



Comparison of the bound amount of different sequences from ZO-1 (2.5 μM superinfused) at the immobilized proteins pointed to a stronger interaction with most of the proteins when the hinge region and the complete SH3-hinge-GuK unit were present. Sequences of ZO-1 (positions see pictograms), full length RGS5 and $G\alpha_{12}$ were N-terminal MBP-fusion proteins. The sequence of ahnak1$^{4893-5535}$ and the complete β2-subunit of L-type Ca^{2+}-channels contained N-terminal GST; occludin$^{406-521}$ (coiled coil-domain) and $G\alpha_{i2}$ were the recombinant wild-type proteins. Mean ± SEM, n ≥ 4; MBP, maltose binding protein; GST, glutathione-*S*-transferase; RU, resonance units. MBP served as negative and occludin as positive control [Schmidt et al., 2001]. Negative values are considered as no binding.
The scheme in the table depicts ZO-1's general domain organization and binding partners of the domains. For the SH3- and GuK-domains, there is a crystal structure [Lye et al., 2010], but not for the hinge region (dotted line). By structural similarity to the hinge of other membrane-associated guanylate kinase homologue proteins (MAGUK), for which a crystal structure exists [McGee et al., 2001], the hinge of ZO-1 is thought to be extended away from the SH3- and GuK-domains and to be freely accessible to interact with other proteins.
[a]Mean ± range, n = 2.
[b]1457 ± 502 ΔRU/4 min.
[c]626 ± 8 ΔRU/4 min.
[d]981 ± 6 ΔRU/4 min.

interaction partners [Taliana et al., 2005]. The actin cytoskeleton adheres to the acidic region of ZO-1 [Fanning et al., 2002]. SH3- and GuK-domains are held together by a flexible hinge region, forming a structural and functional SH3-hinge-GuK unit. In this unit, the hinge region and the GuK-domain bind to the TJ protein occludin or to the adherens junction protein α-catenin [Mueller et al., 2005]. The structure and functional relevance of the hinge is still unclear. However, it has been shown to be phosphorylated [Balda and Matter, 2000] and, thus, a regulatory role is proposed. As the regulatory mechanism of the TJ function is an unsolved question, the elucidation of the structure and function of the hinge region therefore is of general interest.

TJ can be influenced by G-protein signaling. $G\alpha_o$ may stimulate and $G\alpha_i$ may inhibit adenylyl cyclases forming kinase (PK) A phosphorylating, for instance, claudin-5 [Soma et al., 2004]. Claudin-5 seals the TJ, for example, in the blood–brain barrier [Nitta et al., 2003], which are still more tightened after the phosphorylation. G-protein βγ-complexes can influence cellular Ca^{2+} fluxes via phospholipase C and PKC which, in turn, phosphorylates occludin and regulates TJ [Andreeva et al., 2006]. $G\alpha_o$ activation accelerates TJ biogenesis [Denker et al., 1996]. $G\alpha_{i2}$ supports TJ development; its activation increases paracellular tightness [Saha et al., 1998]. In kidney epithelium, $G\alpha_{12}$ colocalizes with ZO-1; transfection of constitutively active mutant $QL\alpha_{12}$ reduces the tightness [Meyer et al., 2002]. The regulator protein of G-protein signaling 5 (RGS5) is found in brain endothelial TJ [Lippoldt et al., 2001], binds $G\alpha_{i1-3,o,q}$, and may inhibit G-protein-dependent transient intracellular Ca^{2+}-release by endothelin [Zhou et al., 2001]. The multifunctional, giant protein ahnak1, earlier described as desmoyokin in desmosomes, translocates between nucleus and plasma membrane in epithelial cells [Sussman et al., 2001]. It is found in enlargeosomes where it colocalizes with ZO-1 [Cocucci et al., 2004]. Membrane localization of ahnak1 is accompanied by tightening cerebral endothelial cell clefts [Gentil

et al., 2005]. Ahnak1 associates with the L-type Ca^{2+}-channel β2-subunit belonging to MAGuKs. The association requires the presence of the SH3- and GuK-domain in the β2-subunit and is diminished by phosphorylation of ahnak1 [Haase, 2007].

Taken together, this information suggests colocalization of some of the aforementioned proteins with ZO-1. In addition, the fact that the crystallized hinge of an homologous MAGuK protein, PSD-95, is the adaptor region for signaling proteins [McGee et al., 2001] let us assume that this region is freely accessible in ZO-1 to interact with other proteins. Concluding the literature data and the considerations made, we hypothesized that the hinge region of ZO-1 plays a key role in regulating cell–cell contact proteins, and that signal proteins directly associate and regulate the hinge region and, hence, cell contacts. Modulation of the interactions is postulated to occur by phosphorylation of the hinge region and activation of the regulators. Comparison of the hinge segment between the SH3- and GuK-domain of different MAGuK proteins shows that the hinge region in ZO-1 is highly unique with respect of its amino acid sequence as well as its exceptional length.

Thus, our aim was first to demonstrate that a direct association exists between signal proteins and ZO-1, then, to elucidate the interaction area, and, finally, to show regulation of the interactions. For the first time, we demonstrate a direct binding of several regulatory proteins, such as G-proteins, G-protein regulators and ahnak1, preferentially, to the hinge region of ZO-1. This binding was influenced by phosphorylation-like modifications of the hinge region and activation of the signal proteins, respectively, in studies of purified proteins but also of living cells.

MATERIALS AND METHODS

CULTIVATION AND TRANSFECTION OF CELLS

Caco (human colorectal adenocarcinoma)-2 and HEK (human embryonal kidney)-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS), 2 mM L-glutamine, and 100 U/ml penicillin, 100 mg/ml streptomycin (cDMEM). Twenty-four hours before transfection, cells were replated on 12 mm glass coverslips covered with poly-L-lysine (Sigma–Aldrich Chemie, Steinheim, Germany) and cultured overnight. The next morning, the medium was replaced with antibiotics-/serum-free DMEM and the cells were transfected by means of Lipofectamine 2000 (supplier's recommendations; Invitrogen, Karlsruhe, Germany), with the cDNA of cyano fluorescence protein (CFP)$G\alpha_{i2}$ or/and ZO-1-yellow fluorescence protein (YFP), or CFP and $G\beta_1\gamma_2$. Medium was exchanged for cDMEM next morning and after 48 h. For transfection, human ZO-117-1692 (NCBI BX640879) was cloned via Cfr9I and *Sal*I sites C terminally fused with YFP (vector pEYFP-N1, BD Biosciences, Freiburg, Germany). To generate (CFP)$G\alpha_{i2}$, $G\alpha_{i2}$ cDNA encoding enhanced CFP (eCFP) was inserted between position 91 and 92 of rat $G\alpha_{i2}$ via two linkers ($G\alpha_{i2}$–1-87-MGNL_*EFMV-YFP-LYSS*_QIDF-$G\alpha_{i2}$–96-355). For generation of the expression plasmid, N-terminal rat $G\alpha_{i2}$ cDNA was amplified using the primers 5′-GGT ACC TGA GAG CTT CCC GCA GAG G-3′ and 5′-GAA TTC CAG GTT GCC CAT GGC TTT GAC GAT GG-3′, introducing restriction sites for *Kpn*I and *Eco*RI. C-terminal rat $G\alpha_{i2}$ cDNA was amplified using the primers 5′-AAG CTT TAC AGC AGC CAG ATC GAC TTT GCT GAC CCC CAG C-3′ and 5′-CTC GAG TAG AGC CGA GGG AGG GGA CAG G-3′; introducing restriction sites for *Hin*dIII and *Xho*I. eCFP cDNA was amplified using the primers 5′-GAA TTC ATG GTT GTG AGC AAG GGC GAG GAG C-3′ and 5′-GCC AAG CTT GTA CAG CTC GTC CAT GCC G-3′, introducing restriction sites for *Eco*RI and *Hin*dIII. eCFP was subcloned into the rat $G\alpha_{i2}$ cDNA via *Eco*RI and *Hin*dIII in pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) and, subsequently, the entire (CFP)$G\alpha_{i2}$ cDNA was subcloned into pcDNA3 (Invitrogen) via *Kpn*I and *Xho*I.

RECOMBINANT PROTEINS AND G-PROTEIN ACTIVATION

If not stated otherwise, human protein coding DNA was cloned via *Bam*HI and *Sal*I sites N terminally fused with maltose binding protein (MBP; vector pMAL-c2X, NEB, Schwalbach, Germany) or glutathione-*S*-transferase (GST; pEGX-4T1, Amersham Bioscience, Freiburg, Germany). Plasmids were used encoding mouse ZO-1 [Schmidt et al., 2004], mouse occludin [Schmidt et al., 2001], mouse $G\alpha_{12}$ [Gohla et al., 1999], $G\alpha_{i2}$ [Gaibelet et al., 1999], RGS5 [Lippoldt et al., 2001], ahnak1 (a.a. 4893–5535 and 5462–5535; NCBI accession number NP_001611), and rabbit cardiac β2-subunit [Hohaus et al., 2002]. Sequences of ZO-1, were mutated by the QuikChange® procedure (Stratagene, Amsterdam, Netherlands; manufacturer's instructions) and used for surface plasmon resonance. MBP- and GST-fusion proteins were overexpressed and purified via affinity chromatography [Schmidt et al., 2004]. For $G\alpha_{i2}$, baculovirus encoding wild type $G\alpha_{i2}$ was coexpressed together with $G\beta_1\gamma_{2\text{-his}}$ in Sf9 cells. Harvested cells were disrupted and cell membrane-extracted heterotrimeric G_{i2} was loaded on Ni-NTA-column; subsequently $G\alpha_{i2}$ was recovered by subunit exchange chromatography using AlF_4^-. $G\alpha_{i2}$ was further purified by anion-exchange chromatography. Purity was assessed by Coomassie-stained SDS-gels, activity by quantitative ^{35}S-GTPγS-binding, and the protein concentration with the Lowry assay (Sigma Diagnostics, St. Louis, MO). The protein was stored at −80°C. G-protein activation was initiated by addition of 10 mM NaF (Sigma–Aldrich Chemie), 50 μM $AlCl_3$ (Omikron, Neckarwestheim, Germany), 10 μM GDP (guanidindiphosphate; Sigma, Munich, Germany), 6 mM $MgCl_2$ (Mallinckodt Baker BV, Deventer, Netherlands), and 2 mM DTT (dithiothreitol; Merck, Darmstadt, Germany); the control was DTT.

IMMUNOCHEMISTRY

Horseradish peroxidase (HRP)-conjugated anti-rabbit immunglobulin G (IgG), HRP-conjugated anti-mouse IgG, Cy3-conjugated anti-mouse IgG (Zytomet, Berlin, Germany), and Alexa-Fluor-488-conjugated anti-rabbit IgG antibodies (Molecular Probes, Eugene) were used. Anti-GFP antibody 01 (Ralf Schülein, Berlin, Germany) was used for CFP and YFP. Antibodies against ZO-1 and occludin were from Zymed (San Francisco), $G\alpha_{12}$ from Santa Cruz Biotechnology (Santa Cruz, Heidelberg, Germany), $G\alpha_{i2}$ from Santa Cruz Biotechnology; RGS5 [Lippoldt et al., 2001]; ahnak1 and β2-subunit [Haase et al., 1999; Hohaus et al., 2002]; α-catenin from Transduction Laboratories (Lexington). Anti-integrin α3 was from Santa Cruz Biotechnology. For immunocytochemistry, Caco-2 cells were washed with PBS (Biochrom, Berlin, Germany), fixed with acetone (5 min, 4°C), washed again with ethanol (1 min, 4°C) and PBS (1 min, 4°C), soaked in blocking solution (BS; 1% bovine serum

albumin and 0.05% Tween 20 in PBS) for 10 min and, then, incubated with the first primary antibody in BS for 1 h. After washing (5 × 2 min BS), samples were incubated with the second primary antibody in BS for 1 h, washed as before, incubated with secondary antibodies for 30 min in BS, and washed again. Cells were examined using an LSM 510 confocal microscope (Zeiss, Jena, Germany).

For coimmunoprecipitation, Caco-2 lysates were frozen in liquid N_2, thawed, scraped off on ice, and passed through a 21 Gauge needle 20×. Briefly, antibodies against $G\alpha_{i2}$, ZO-1, integrin α3, or PBS were incubated with protein-G sepharose 4B fast-flow (Sigma–Aldrich, Steinheim, Germany) for 60 min at RT, centrifuged (3,000*g*, 2 min), and washed 3× with ice-cold PBS. Immediately thereafter, lysates were added to the antibody-sepharose complexes and incubated overnight at 7°C; the immunoprecipitate (3,000*g* centrifugation, 2 min, 4°C) was washed 4× with ice-cold PBS, resuspended in Laemmli buffer, heated (95°C, 10 min) and centrifuged (12,000*g*, 30 s) to pellet down the sepharose beads while recovering the supernatant containing the solubilized proteins. The precipitated proteins were separated by SDS-gel electrophoresis and detected by Western blotting using anti-Gαi2 or anti-ZO-1 antibodies.

SURFACE PLASMON RESONANCE SPECTROSCOPY (SPR)

A Biacore 2000, CM4 and CM5 chips (research grade; BIACORE AB, Uppsala, Sweden) were used according to [Schmidt et al., 2004]. Occludin$^{406-521}$ (cytosolic C-terminal coiled coil-domain), MBP-$G\alpha_{12}$, $G\alpha_{i2}$, MBP-RGS5, GST-ahnak1$^{4893-5535}$, GST-β2-subunit, and MBP were immobilized on the chip according to the manufacturer's instruction using 10 mM Na-acetate buffer pH 4–6. For better comparison, the superfused ZO-1 constructs were normalized to their molecular weight and the associated amount was expressed as RU (resonance units). The binding measurement was carried out by 4 min superfusion (0.5–10 μM) of the ZO-1 fragments (flow rate 8 μl/min; running buffer: PBS, 5 mM maltose). If not stated otherwise, MBP-ZO-1$^{516-806}$ was constructed to represent the SH3-hinge-GuK unit (ZO-1$^{516-806}$), MBP-ZO-1$^{502-576\sim631-645}$ (without a.a. 577–630) the SH3-domain (ZO-1$^{516-575}$), and MBP-ZO-1$^{644-772}$, the GuK-domain (ZO-1$^{644-794}$). MBP-ZO-1$^{502-812}$ (including complete SH3-hinge-GuK unit), $^{413-619}$ (SH3), and $^{644-890}$ (GuK) were also tested. Negative controls were the corresponding concentrations of MBP. If not stated otherwise, means ± SEM are given.

PEPTIDE EPITOPE MAPPING

To analyze ZO-1 epitopes, spots with 20-mer peptides spanning ZO-1$^{502-812}$ with a shift of 2 a.a. between each peptide were synthesized on Whatman 50 paper (Maidstone, England) [Frank and Overwin, 1996; Schmidt et al., 2004]. Similarly, 25-mers from ZO-1$^{599-633}$ were mapped. Peptides spotted on the same membranes were analyzed by MS and HPLC to control the synthesis. After 2 h in blocking buffer made from blocking buffer concentrate (Sigma #B6429) with 5% saccharose in Tris buffered saline pH 8.0 (TBS), the membranes were incubated overnight with 200 nM fusion proteins or wild type $G\alpha_{i2}$ in blocking buffer. After 3 × 10 min washing in TBS, the membranes were treated for 90 min with anti-GST-HRP-conjugate [Mueller et al., 2005], anti-MBP-HRP-conjugate or anti-$G\alpha_{i2}$ mouse antibody (1:5,000 in TBS, each; Amersham Bioscience). Secondary antibody for the latter was HRP-conjugated anti-mouse IgG (Santa Cruz Biotechnology). Binding was detected after 3 × 10 min washing in TBS with a chemiluminescence kit (Uptima; Interchim, Montiucon Cedex, France) at Lumi-Imager F1^{TM} (Boehringer, Mannheim/Germany). MBP alone, GST alone (1:1,000; Amersham Bioscience) or anti-mouse IgG were used as the negative control, which was subtracted from the binding values.

QUANTITATIVE COLOCALIZATION ANALYSIS AND EPINEPHRINE TREATMENT

Forty eight hours after cotransfection of CFP-/YFP-fusion proteins, HEK cells were fixed in ice-cold methanol/acetone (v:v, 1:1) and mounted on a coverslip for fluorescence analysis in LSM 510 biphotonic confocal microscope (Zeiss). Colocalization analysis was performed using Zeiss proprietary confocal imaging software and Image J using the colocalization threshold plug-ins (Tony Collins, Wright Cell Imaging Facility, Toronto, Canada). In image J, colocalization processing was implemented [Costes et al., 2004] and a comprehensive colocalization coefficient was calculated: $Ccf = (P_c^2/P_t)/[(CFP_{I>t}/CFP_{It}) \times (YFP_{It}/YFP_{I>t})]$; Ccf, colocalization coefficient; P_c^2, number of colocalizing pixels; P_t, total number of pixels; $CFP/YFP_{I>t}$; sum of total pixel intensities above the intensity threshold in CFP/YFP channel; CFP/YFP_{It}, sum of the total pixel intensities. (ι)-Epinephrine(+)-bitartrate (Sigma–Aldrich) dissolved in PBS (final concentration 5 μM) was added for 10 min; PBS served as control.

RESULTS

COLOCALIZATION OF REGULATORY PROTEINS AND ZO-1 IN EPITHELIAL CELLS

Colocalization of regulator proteins and the scaffolding protein of the contacts ZO-1 was studied in Caco-2 cells forming functioning TJ (Fig. 1). ZO-1 and the TJ protein occludin were solely detectable in the cell membrane where two neighboring cells were in close contact. Both showed complete colocalization as known (Andreeva et al., 2006). RGS5, ahnak1, β2-subunit of L-type Ca^{2+}-channel, and α-catenin also showed colocalization in the cell contacts. For RGS5 and β2-subunit, colocalization was continuous, that is, both were present at those segments in the cell membrane where ZO-1 was. However, the weak expression of RGS5 and β2-subunit might result in an underestimation of their fluorescence relative to that of ZO-1 in the merge. For ahnak1 and α-catenin, the staining overlapped discontinuously. This means that neither ahnak1 nor α-catenin was located in all parts of the cell membrane where ZO-1 was found; additionally, ahnak1 and catenin formed submembranous bands. The colocalizations were confirmed in MDCK cells (not shown). Moreover, Figure 1 shows weak fluorescence of $G\alpha_{12}$ and, in part, of $G\alpha_{i2}$ in contact areas between Caco-2 cells with colocalization of ZO-1. Both G-proteins exhibited staining in the cell membrane and in the cytosol. For MDCK cells and $G\alpha_{i2}$, although there was little expression in the cell membrane, weak colocalization with ZO-1

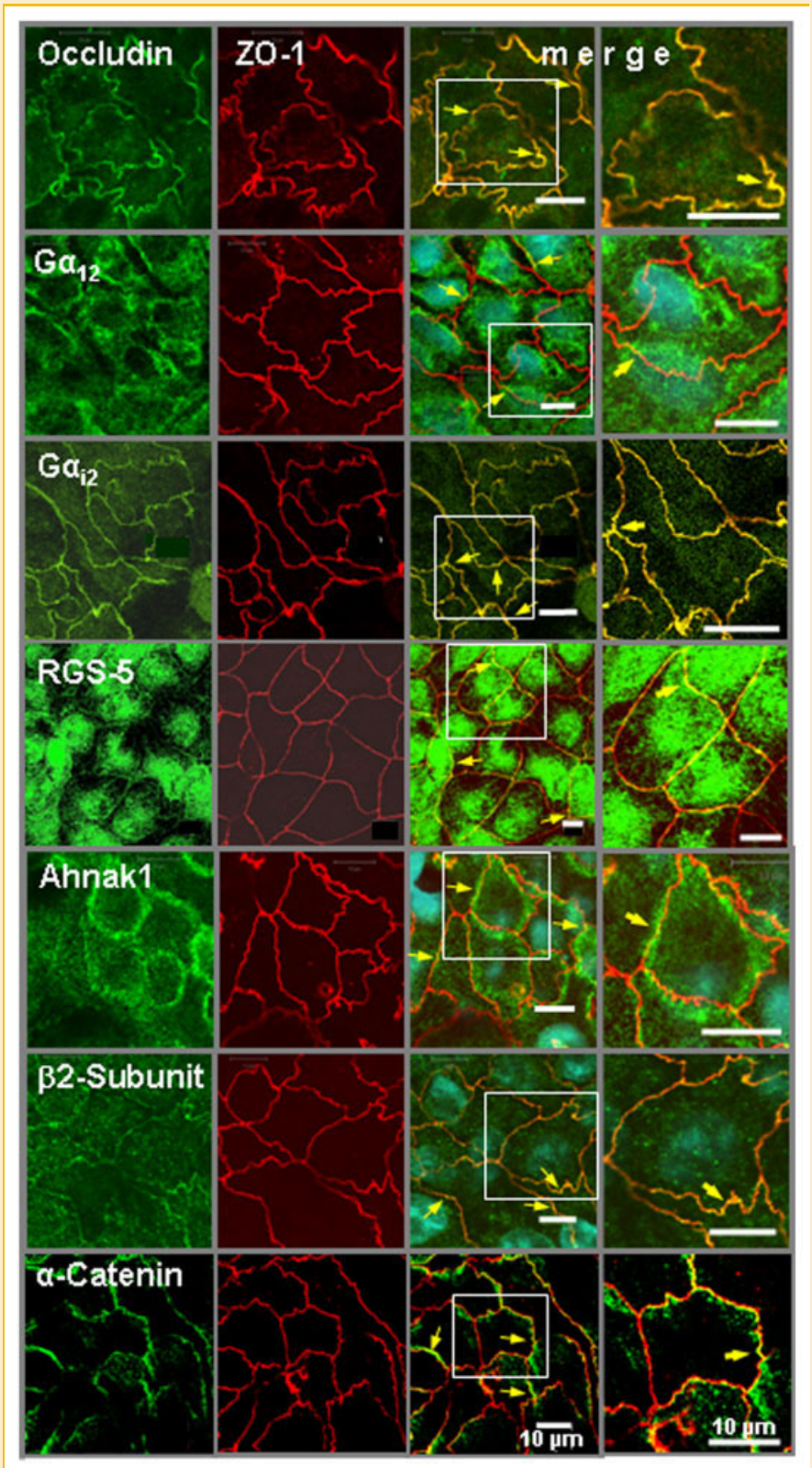


Fig. 1. Coimmunostaining of proteins with potential regulatory relevance (green) for cell–cell contacts and of ZO-1 (red) in confluent Caco-2 cells. In the merge modus, occludin, RGS5, ahnak1, β2-subunit, and α-catenin showed colocalization (yellow to orange) with ZO-1 in the plasma membrane (arrows). With $G\alpha_{12}$ and $G\alpha_{i2}$, no considerable overlap was found with ZO-1. Bars, 10 µm.

cannot be excluded, as a slight change in the fluorescence color in the merge modus was denoted (not shown).

ASSOCIATION OF ZO-1 SH3-HINGE-GUK UNIT AND REGULATORY PROTEINS

To demonstrate the molecular interactions between potentially regulatory proteins and ZO-1, the association between recombinant proteins out of them was investigated by SPR. Table I revealed that the proteins tested associated with the SH3-hinge-GuK unit of ZO-1 (ZO-$1^{516-806}$). A sequence covering the SH3-domain alone (ZO-$1^{502-576\sim631-645}$) did not show notable binding values except in the case of RGS5 and of the positive control occludin. The measurements indicated two types of interaction. In the 'unit type' (for occludin, G proteins, RGS5, ahnak1), the complete SH3-hinge-GuK unit exhibited stronger adherence than the SH3- or GuK-domain alone. The 'domain type', was observed for the β2-subunit, characterized by similar binding intensity for the GuK-domain (ZO-$1^{644-772}$) and complete unit. The interactions with the complete unit were concentration dependent for occludin [Schmidt et al., 2001], $G\alpha_{12}$, $G\alpha_{i2}$ (500 nM–10 μM), for RGS5, ahnak1, and β2-subunit (1–10 μM). The binding values of ZO-$1^{516-806}$ (0.5, 1, 2.5, 5, 10 μM) were 161 ± 32, 243 ± 48, 626 ± 15, 915 ± 86, 3200 to $G\alpha_{12}$; 309 ± 56, 389 ± 46, 981 ± 12, 1407 ± 92, 4593 to $G\alpha_{i2}$; 96 ± 15, 106 ± 13, 338 ± 61, 895 ± 89, 1869 ± 80 to RGS5; not detected, 143, 299 ± 9, 913, 1398 to ahnak1; 58, 92, 515, 907, 1679 to β2-subunit (measurement in ΔRU/4 min; mean values ± SD if $n = 4$).

The associations were assumed not to be due to the MBP or GST, used as tag for some of the recombinant proteins. MBP did not interact appreciable with ZO-1 constructs (Table I). GST also does not interact with ZO-1 [Meyer et al., 2002] and did not influence the interactions studied essentially as, for example, the association of 5 μM ZO-$1^{516-806}$ to immobilized GST-ahnak$1^{4646-5288}$ resulted in 767 ΔRU/4 min which is similar to 651 ΔRU/4 min measured to immobilized His_6-ahnak$1^{5215-5288}$ the binding segment of ahnak1 [Haase et al., 2005]. Moreover, it can be assumed that the signaling proteins, after the fusion, are freely accessible and the adherence of ZO-1 is not affected. This accessibility is the case for ahnak1 as both the GST- and His-tag allowed association in a similar interaction range. For the G-proteins, the fusion protein did not seem to have an influence as MBP-$G\alpha_{12}$ was activated and bound in the same manner as the tag-free $G\alpha_{i2}$. In preliminary experiments, ZO-1 was coimmunoprecipitated from MDCK cells with GST-RGS5, which was impossible with GST alone. This data indicated free accessibility also in the case of RGS5. For the GST-β2-subunit, it was shown that the binding to ahnak1 is related to the subunit only but not to GST alone [Hohaus et al., 2002].

When the SH3-domain (ZO-$1^{516-575}$) was C terminally prolonged by sequences of the hinge region (ZO-$1^{576-643}$), binding intensity to the regulatory proteins became higher than to the SH3-domain alone (latter see the first column of Table I, values $\ll 2.7$ ΔRU/mol × 4 min, except occludin 7.3). Thus, ZO-$1^{502-645}$ (covering SH3-domain plus hinge region) bound (in ΔRU/mol × 4 min): 12.61 ± 1.31 to occludin, 5.00 ± 0.20 to $G\alpha_{12}$, 7.41 ± 0.25 to $G\alpha_{i2}$ or 2.97 to the β2-subunit. A very similar sequence of SH3-hinge, ZO-$1^{502-645\sim795-806}$, bound to RGS5 with 3.52 ± 0.10 and to ahnak$1^{4893-5535}$ with 4.22 ± 0.25 ΔRU/mol × 4 min.

PREFERRED BINDING OF REGULATORY PROTEINS TO THE HINGE REGION OF ZO-1

The association of different ZO-1 domains, including the complete SH3-hinge-GuK unit, superinfused to immobilized occludin, $G\alpha_{12}$, $G\alpha_{i2}$, RGS5, and ahnak1 (Table I), was supported by reverse experiments (Fig. 2) which led to comparable results. In these peptide-mapping studies, we immobilized peptide sequences out of the SH3-hinge-GuK on a membrane and demonstrated binding of the regulatory proteins with the membrane. This assay, mapping the binding area(s) in ZO-1, showed that the different proteins associated preferentially to nearly the same sequences within the hinge region (ZO-$1^{576-643}$, dashed lines in Fig. 2). For the positive control occludin, this area was within ZO-$1^{592-623}$, for $G\alpha_{12}$ within ZO-$1^{584-619}$, for $G\alpha_{i2}$ within ZO-$1^{604-629}$, for RGS5 within ZO-$1^{598-625}$, and for ahnak1 within ZO-$1^{606-631}$ (arrows, solid lines). For RGS5 and ahnak1, an additional binding area was observed in the GuK-domain (ZO-$1^{644-794}$), within ZO-$1^{756-781}$ for RGS5, and within ZO-$1^{726-751}$ for ahnak1 (Fig. 2), corresponding to predicted [Mueller et al., 2005] α-helices ZO-$1^{747-768}$ and ZO-$1^{732-740}$.

The preferred binding capacity of the hinge region was supported by SPR measurements in which MBP-$G\alpha_{12}$ and $G\alpha_{i2}$ were immobilized and segments of ZO-1 were superfused in 2.5 μM concentrations. Concerning the association to $G\alpha_{12}$, 665 ± 21 ΔRU/4 min were registered for the central element of the hinge region ZO-$1^{597-633}$ (synthesized peptide). This value is comparable with the complete SH3-hinge-GuK unit (MBP-ZO-$1^{516-806}$, 626 ± 15 ΔRU/4 min) and considerably higher than for the GuK-domain alone (MBP-ZO-$1^{644-772}$, 204 ± 4 ΔRU/4 min) or the SH3-domain alone (MBP-ZO-$1^{502-576\sim631-645}$, no binding detectable). For $G\alpha_{i2}$, the association of the hinge (ZO-$1^{597-633}$) was 674 ± 11 ΔRU/4 min, which was less for GuK alone (MBP-ZO-$1^{644-772}$, 347 ± 17 ΔRU/4 min) or SH3 alone (MBP-ZO-$1^{502-576\sim631-645}$, no binding). However, the complete unit showed still better values for the binding of $G\alpha_{i2}$ (MBP-ZO-$1^{516-806}$, 981 ± 12 ΔRU/4 min) than the hinge did.

PHOSPHORYLATION IMITATION IN ZO-1 HINGE REGION AND G-PROTEIN ACTIVATION STIMULATED THEIR INTERACTION

Figure 2 also shows that all peptides of the predicted coiled coil-helix (ZO-$1^{600-624}$) in the hinge region strongly associated with $G\alpha_{12}$, $G\alpha_{i2}$, RGS5, and ahnak1, and share the same sequence: GLR*SS* KRNLR KSRE (ZO-$1^{606-619}$). This sequence contains serines[609] and [610], which can be phosphorylated [Balda and Matter, 2000]. We therefore performed SPR binding measurements with the complete SH3-hinge-GuK unit (ZO-$1^{516-806}$) with the serines replaced by glutamate mimicking phosphorylation. Figure 3B reveals that the superfused mutant ZO-$1^{516-806}{}_{S610E}$ showed 3.5-fold higher binding to the immobilized G-proteins α_{12} and α_{i2} than the respective wt sequences (Fig. 3D). The associations were concentration-dependent (sub- and low-micromolar), and linear. The same enhancement was observed with the ZO-$1^{516-806}{}_{S609E}$ on both G-proteins (not shown).

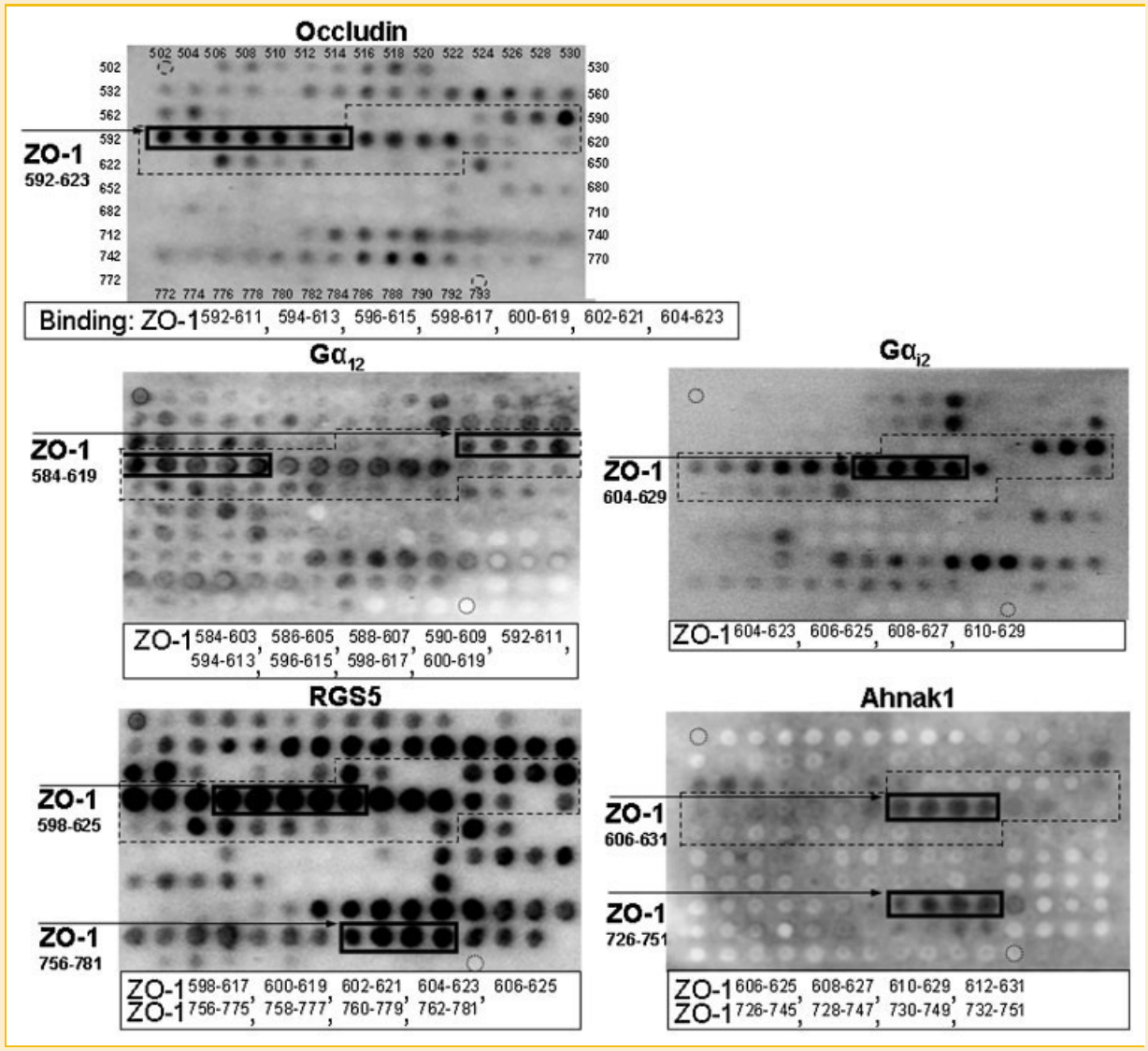


Fig. 2. Peptide mapping binding assay of occludin$^{406-521}$, Gα_{12} (N-terminal MBP-fusion proteins), RGS5, ahnak1$^{4893-5535}$ (N-terminal GST-fusion proteins), and Gα_{i2} incubated on immobilized peptides of the SH3-hinge-GuK unit of ZO-1 revealing preferred binding at sequences within the hinge region of ZO-1 (ZO-1$^{576-643}$, dashed lines). As positive control, the known binding of occludin to the center of the hinge region (solid line) [Schmidt et al., 2004] is shown. Each spot contained 20-mer peptides of ZO-1; numbers in upper panel mark the N-termini of the respective 20-mers; dotted circles mark first and last spot synthesized on the membrane. Solid lines enframe, at least, four consecutive spots with $\geq$50% binding intensity, compared to the spot with maximum binding, measured in two experiments; ZO-1 numbers at arrows indicate first and last amino acid of the sequences contained in those spots framed by solid lines. Optical density of a spot corresponded to the binding intensity measured as fluorescence due to horseradish peroxidase (HPR) linked anti-MBP, anti-GST antibodies, or anti-mouse IgG (for anti-Gα_{i2}). MBP alone, GST alone, or anti-mouse IgG were used as negative control which was subtracted from the respective fluorescence values.

In addition to the ZO-1 modification, we studied the modulation of regulatory proteins. Thus, Figure 3C displays that ZO-1$^{516-806}{}_{wt}$ (SH3-hinge-GuK) bound better to Gα_{12} and Gα_{i2} after G-protein activation than the control (Fig. 3D). This association followed saturation kinetics with maximum increases of 5× for Gα_{12} and 4× for Gα_{i2}, when 2.5 μM ZO-1 construct was superfused. The same kinetics with the same concentration maximum, but 6× and 5× binding intensification, respectively, were registered when both mutant ZO-1$^{516-806}{}_{S610E}$ and G-protein activation were investigated simultaneously (Fig. 3A). A similar enhancement was observed with ZO-1$^{516-806}{}_{S609E}$ plus activated G-proteins (raised 5×, not shown).

Similar binding data were obtained with an inverse approach using peptide mapping (Fig. 4). Here, 25-mer peptides out of the coiled coil-domain in the hinge (ZO-1$^{600-624}$), as well as mutants of these peptides were immobilized on membrane spots which, then, were incubated with Gα_{12}. The G-protein binding was considerably increased when phosphorylation-mimicking mutants were compared with the wild type. The strongest interaction was found when the serines were replaced by glutamate (S609E, S610E, S609E/S910E) and when the whole coiled coil-sequence was present. All peptides exhibiting binding of Gα_{12} shared the same sequence, RFR GLR*SS* KRNLR KSRED LSA (ZO-1$^{603-623}$), right in the center of the

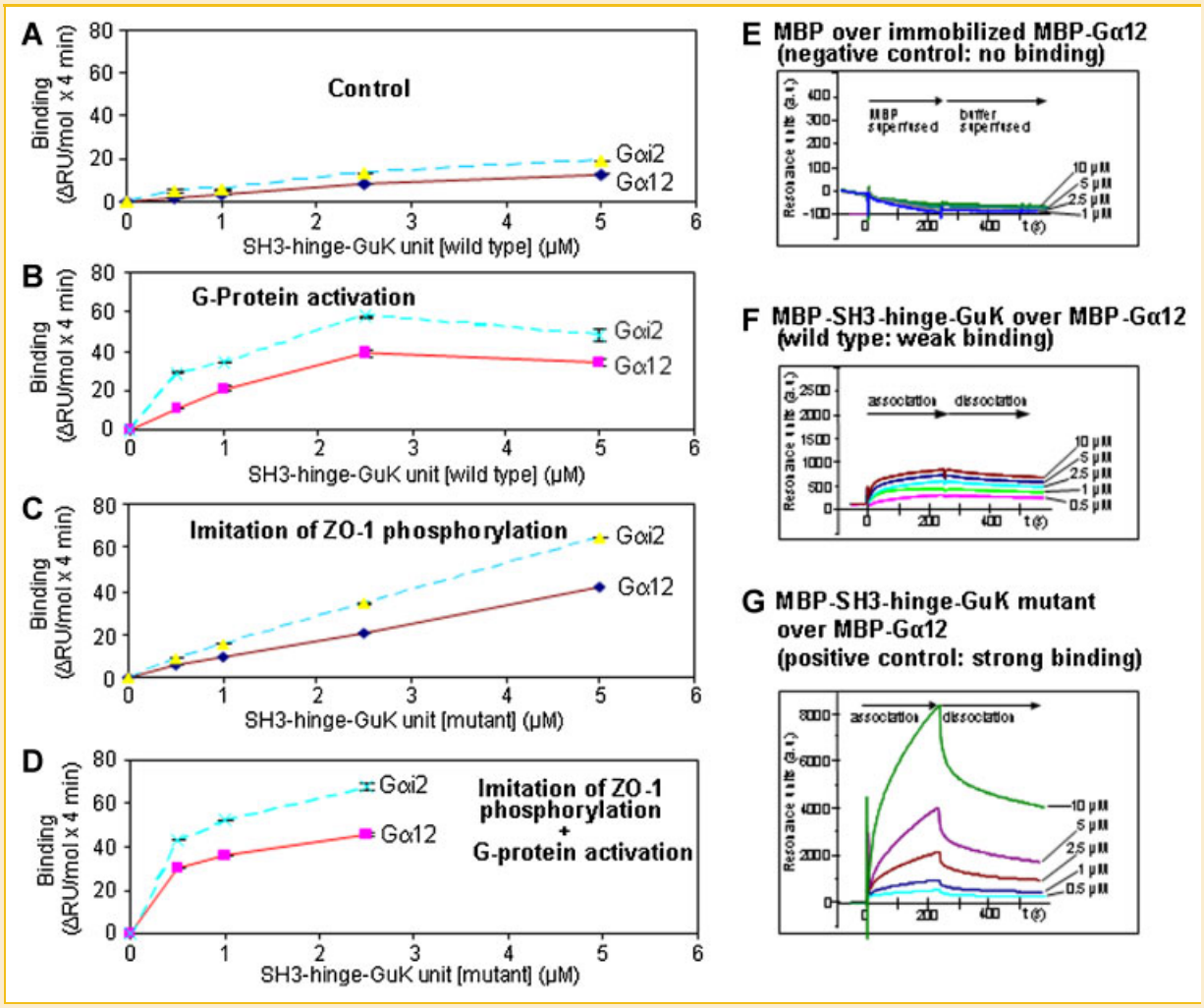


Fig. 3. Manipulation of the interaction between complete SH3-hinge-GuK unit of ZO-1 and Gα-proteins as measured by surface plasmon resonance spectroscopy. A: MBP-ZO-$1^{516-806}$ was superfused over the immobilized G-proteins $G\alpha_{12}$ and $G\alpha_{i2}$ in running buffer; (B) for activation of G-proteins, 10 mM NaF, 50 μM $AlCl_3$, 10 μM GDP, 6 mM $MgCl_2$, and 2 mM DTT were added to the buffer. C: To mimic phosphorylation in the hinge region of ZO-1 the mutant ZO-$1^{516-806}{}_{S610E}$ was used in which serine 610 was replaced by glutamate. D: The effect of the G-protein activation and the imitation of ZO-1 phosphorylation, when applied simultaneously. Binding data are expressed as ΔRU/mol during 4 min of association and represent mean ± SEM, n = 4. E,F: show original sensograms of MBP, as negative control, the weak binding of MBP-ZO-$1^{516-806}$ wild type, and MBP-ZO-$1^{516-806}$ mutant, as positive control, over immobilized MBP-$G\alpha_{12}$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

hinge region. As S-E replacement in the hinge may not totally reconstitute the effect of phosphorylation, as positive control, we tested occludin$^{406-521}$ which is well-known to interact with ZO-1. Occludin bound less to 30-mer peptides of both the phosphorylation mimic ZO-1_{S610E} (1.7 ± 0.2 a.u.) and the phosphoserine ZO-1_{S610P} (2.2 ± 0.2 a.u.) than to the wild type ZO-1_{S610} (2.5 ± 0.3 a.u., $P < 0.001$ and 0.05, respectively, n = 22). This difference demonstrates that the glutamate mutant mimicked the phosphorylation.

$G\alpha_{i2}$ CAUSED MEMBRANE LOCALIZATION AND COLOCALIZATION OF ZO-1

The direct association of regulator proteins to the SH3-hinge-GuK unit found at the protein level (Table I; Figs. 2–4) could be facilitated via signal transduction in the cellular environment. For this intention, the interaction between the C-terminal YFP-fusion protein of ZO-1 (ZO-1-YFP) and (CFP)$G\alpha_{i2}$ was estimated after co-transfection and epinephrine stimulation in HEK cells. Figure 5, upper panel, shows that only a little of the G-protein was at the cell membrane, whereas the majority was in the cytosol. After epinephrine activation of the cells, a part of the G-protein signal was enriched in the plasma membrane (arrows). Moreover, the activated G-protein better colocalized with ZO-1, as indicated by the increase of the colocalizing pixels after merging the images (middle panel, right). The addition of epinephrine did not influence the plasma membrane distribution of ZO-1 to any great extent (compare left images, upper and middle panel). The colocalization of $G\alpha_{i2}$ to ZO-1 caused by 5 μM epinephrine was accompanied by an increase in the colocalization coefficient from 0.95 ± 0.15 to 5.20 ± 1.19, $P < 0.01$, n = 25. However, HEK cells monotransfected with ZO-1-YFP mainly showed intracellular fluorescence (Fig. 5, lower panel, left), which

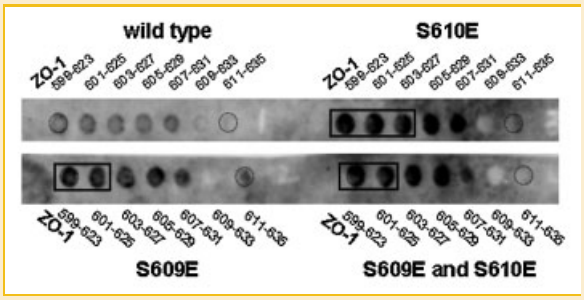


Fig. 4. Phosphorylation-like modification on serine^{609} and/or serine^{610} (replaced by glutamate) in the predicted coiled coil-helix [Mueller et al., 2005] within the hinge region of ZO-1 intensified the attraction of N-terminal MBP-fusion protein of $G\alpha_{12}$ compared to the wild type helix. Each spot contained a 25-mer peptide of ZO-1; dotted circles mark first and last spot of a series of peptides; numbers above or below a membrane mark the 25-mers. Solid lines enframe, at least, two consecutive spots with $\geq$50% binding intensity, compared to the spot with maximum binding, taken as means from two experiments. Optical density of a spot corresponded to the binding intensity measured as fluorescence due to horseradish peroxidase linked anti-MBP antibodies.

moved to the cell membrane only after cotransfection with $G\alpha_{i2}$ (upper panel, left). Monotransfection with (CFP)$G\alpha_{i2}$ also resulted in intracellular localization which was directed to the plasma membrane only after activation by cotransfection with $G\beta_1\gamma_2$. CFP remained intracellular, even if coexpressed with $G\beta_1\gamma_2$ (lower panel, right).

$G\alpha_{i2}$ AND ZO-1 INTERACTION IN CELLS

In Caco-2 lysates, coimmmunoprecipitation using $G\alpha_{i2}$ antibodies demonstrated binding of ZO-1 to $G\alpha_{i2}$ (Fig. 6, lane 1). The converse experiment with ZO-1 antibodies demonstrated binding of $G\alpha_{i2}$ to ZO-1 (Fig. 6, lane 2). Without the antibodies, no association was detected (Fig. 6, lanes 3, 4). Moreover, antibodies against integrin α3, which is unknown to interact with ZO-1, also did not demonstrate association (Fig. 6, lanes 5, 6).

DISCUSSION

The results demonstrate, for the first time, direct interactions between ZO-1 and potentially regulatory proteins with a wide variety of structures and functions. ZO-1 is the scaffolding protein of various cell–cell contacts, differing in molecular composition, and cellular function. The hinge region within the SH3-hinge-GuK unit of ZO-1 was identified as the major adapting area. This means that the hinge region has to be exposed, accessible, and flexible to bind these structurally different regulators. Thus, the hinge region might connect diverse signal transduction elements and pathways, respectively, with cell-cell contacts. The flexibility is presumed from X-ray structure investigation of the SH3-hinge-GuK unit. The hinge region did not show a diffraction signal, it means this part is not aligned and should be flexible in solution. One supposed therefore that the region is flexible [Lye et al., 2010]. Moreover, the crystal structure of a heterodimeric SH3-GuK construct [Lye et al., 2010] supports our finding that SH3-domain, hinge region, and GuK-domain form a structural and functional unit [Mueller et al., 2005].

The association between the regulatory proteins was detected by independent techniques: SPR, peptide mapping and coimmunoprecipitation. Thus, comparable data have been obtained, independently of whether ZO-1 or regulatory proteins were immobilized or incubated, and whether peptide sequences, whole domains or complete proteins were studied. In addition, the associations found with recombinant proteins of occludin, α-catenin [Mueller et al., 2005], RGS5, ahnak1 or the β2-subunit of Ca^{2+}-channels are consistent with our observations in cells. After coimmunostaining, the proteins colocalize with ZO-1 in the cell membrane between adhering cells. Colocalization is not a proof of protein binding. However, the proteins simultaneously localize in the cell contacts, which is a precondition for protein interactions and is in agreement with the binding measurements performed.

Without stimulation of Caco-2 cells, the G-proteins studied exhibited stronger staining in the cytosol than in the plasma membrane and, consequently, colocalization with the membrane-associated protein ZO-1 is weak. Nevertheless, association of both proteins is demonstrated by coimmunoprecipitation. In addition, we already found submicromolar concentrations of G-proteins interacting with submicromolar ZO-1 when studying recombinant proteins. The association is considerably intensified after G-protein activation. Cotransfected in HEK cells, we show colocalization of ZO-1 and $G\alpha_{i2}$ in the cell membrane, which is significantly intensified after epinephrine stimulation. This observation is in agreement with the binding studies mentioned above. In cells, colocalization is elicited in the plasma membrane when the G-protein has been activated by incubation with the activator epinephrine of the G-protein coupled β-adrenergic receptor [Pozgajova et al., 2006]. Similarly, coexpression of $G\alpha_{i2}$ and $G\beta\gamma$, also activating G-proteins [Rebres et al., 2011], shifted the G-protein to the plasma membrane. Also, coexpression of ZO-1 and $G\alpha_{i2}$ in TJ-free HEK cells shifted the ZO-protein to the membrane, whereas, we found monotransfected ZO-1 rather intracellularly. Taken together, these experiments show a clear interaction between ZO-1 and the G-protein, which improves localization in the TJ and which is intensified by activation of the G-protein.

Recently, we developed a molecular and structural model which showed that the SH3-domain (ZO-1$^{516-575}$), hinge region (ZO-1$^{576-643}$), and GuK-domain (ZO-1$^{644-794}$) do not act as separate structures but as a single functional unit [Mueller et al., 2005]. This model is supported by both the crystal structure of a hinge-free SH3-GuK construct [Lye et al., 2010] and our results, which demonstrate that a complex is formed. The regulatory proteins, for example, $G\alpha_{12}$ and $G\alpha_{i2}$, RGS5 and ahnak1, exhibit the strongest binding if the complete SH3-hinge-GuK unit is present, but not just a single element. SH3 or GuK alone exhibit less association than the complete unit, or no association at all. These findings point to a specific function of the hinge.

Thus, the hinge might act as regulator and may enhance the association efficiency of the other domains in the unit. Similar effects have already been indicated in studies with the adherens junction protein α-catenin [Mueller et al., 2005] or the TJ protein

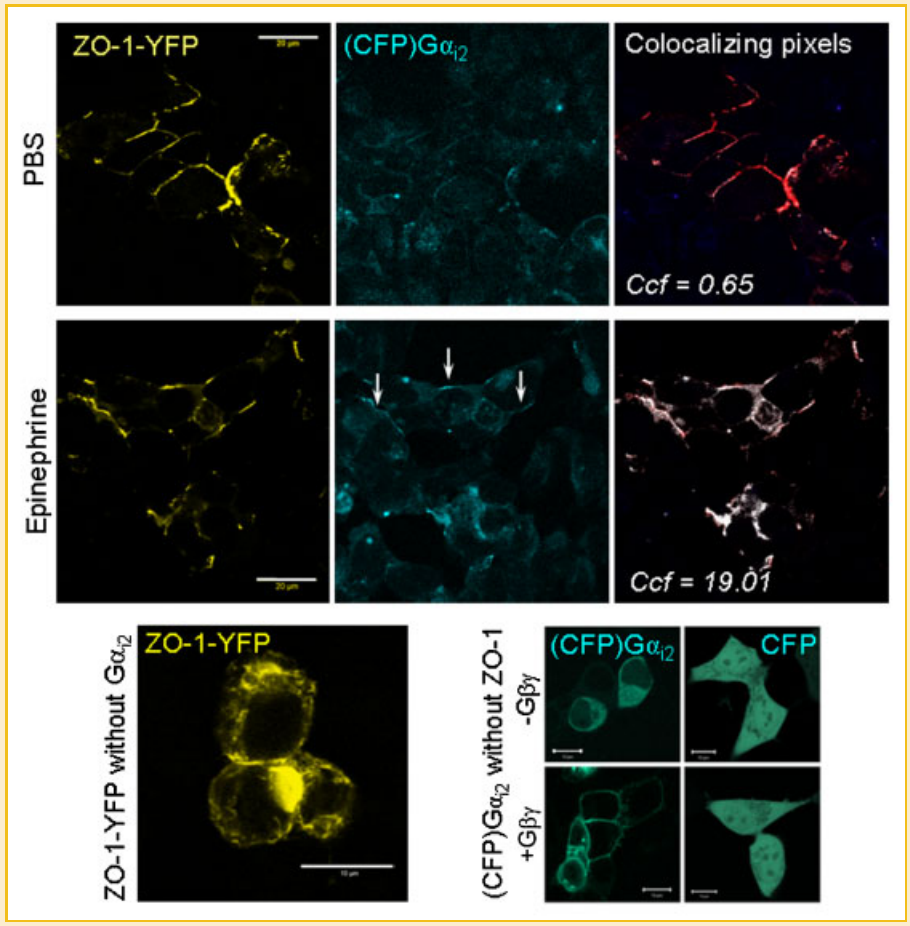


Fig. 5. 10 min incubation of 5 μM epinephrine shifted the intracellular distribution of $G\alpha_{i2}$ from cytosol to the cell membrane (arrows) and caused colocalization (white pixels, right) with ZO-1, when cotransfected in HEK-293 cells (middle panel compared to the upper panel). The images show yellow fluorescence protein (YFP) fused to ZO-1 C-terminally (yellow). $G\alpha_{i2}$ (cyan) contained CFP between the amino acid 91 and 92. Ccf, colocalization coefficient calculated according to [Costes et al., 2004]; scale bar, 20 μm. In addition, ZO-1 shifted from the intracellular compartment (lower panel, left; monotransfection without $G\alpha_{i2}$) to the cell membrane when cotransfected with $G\alpha_{i2}$ (upper panel, left). Similarly, $G\alpha_{i2}$ was only intracellularly in the absence of ZO-1. In addition, (CFP)$G\alpha_{i2}$ accumulated at the cell membrane when $G\beta1\gamma2$ was coexpressed whereas the intracellular localization of CFP alone did not change in the presence of $G\beta1\gamma2$. Scale bars, 10 μm (lower panel, right).

occludin. In the latter, the coiled coil-domain of occludin also showed higher binding values to a hinge-GuK construct than to the GuK-domain alone [Schmidt et al., 2004]. Moreover, it can be assumed that the SH3-hinge-GuK unit is free in the total ZO-1 and is not affected by its other domains. Thus, for instance, both full length ZO-1 and ZO-1 lacking the PDZ-domain(s) may associate occludin in the same manner [Fanning et al., 1998]. Concerning the SH3-hinge-GuK unit, we cannot judge whether SH3- and GuK-domain are able to act as separate structures or as a unit only. On the one hand, the crystal structure of SH3-GuK (without the hinge) [Lye et al., 2010] shows an interface between the two domains which seems to be unable for further protein interaction. On the other hand, the hinge region, as discussed above, is assumed to be an exposed, accessible and flexible part between the domains. This constellation might allow that greater patches of the domain surfaces could interact in a separate manner. However, the hinge region's interactions are not thought to act separately.

A regulatory role of binding proteins on ZO-1 has been suggested for the transcription factor ZONAB. This factor binds to ZO-1, controlling the expression of the ErbB-2 promoter and paracellular permeability. The association sequence is human ZO-$1^{496-604}$ [Balda and Matter, 2000] (equivalent to our mouse ZO-$1^{508-616}$). This sequence contains the SH3-domain and the majority of the hinge region, including the phosphorylation site S^{609}–S^{610} within the hinge. Such a phosphorylation has direct structural and functional consequences [Balda et al., 1996].

The hinge region of the MAGuK protein ZO-1 is unique. The sequence is much longer than 30 other MAGuKs and not homologous to these [Mueller et al., 2005]. Thus, a greater variety of proteins should be capable of adapting to ZO-1. In other MAGuKs, the hinge often acts as target for a specific regulatory adaptor protein, which regulates their structure and function. Thus, calmodulin modulates SAP-102 in regulating neurotransmitter receptors [Masuko et al., 1999] or peptide ligands regulate the oligomerization of PSD-95 in the synaptosomal membrane [McGee et al., 2001]. In ZO-1, the association of occludin to the SH3-hinge-GuK unit is thought to result in swapping of its intramolecular SH3-GuK interaction to an intermolecular SH3-GuK interaction

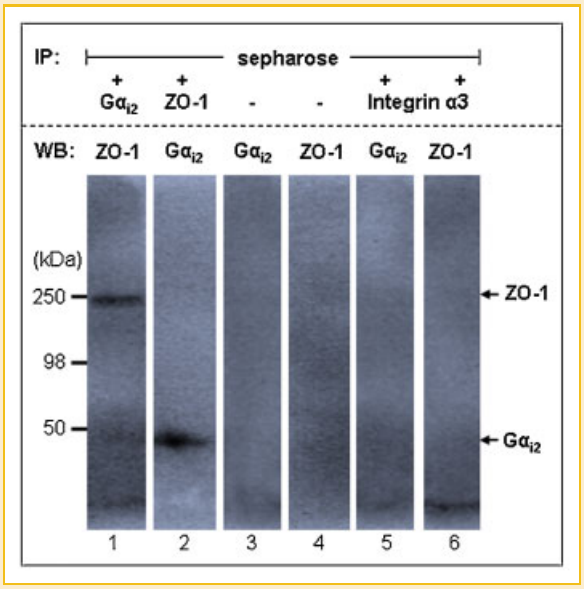


Fig. 6. Association of ZO-1 and $G\alpha_{i2}$ in cells shown by coimmunoprecipitation (IP) of ZO-1 by means of $G\alpha_{i2}$ (lane 1) or $G\alpha_{i2}$ by ZO-1 (lane 2), visualized by Western blotting (WB). Lysates of Caco-2 cells were subjected to sepharose beads preincubated with anti-$G\alpha_{i2}$- and anti-ZO-1 antibodies, respectively. The controls without preincubation did not show IP. Sepharose without anti-ZO-1- (lane 3) or anti-$G\alpha_{i2}$ antibody (lane 4) were free of $G\alpha_{i2}$ or ZO-1, respectively, in the WB similar as the negative controls using beads preincubated with the non-related antibody anti-integrin α3 (lanes 5 and 6) which did not precipitate any of the proteins. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

which supports the dimerization of ZO-1 [Mueller et al., 2005]. Occludin also forms dimers [Walter et al., 2009] and dimers are predicted to interact with the unit [Mueller et al., 2005]. We therefore assume that the hinge in ZO-1 has a regulatory role, based on the structure and function of its SH3-hinge-GuK unit and which pertains to cell–cell contacts. This assumption is supported by the observation that the hinge is sufficient for occludin binding and required for targeting ZO-1 to TJ [Fanning et al., 2007].

The hinge region is highly conserved between ZO proteins and between different species, that is, >90% identity and >95% functional similarity. The data obtained in this study, therefore, can be transferred to other ZO proteins and may explain why cells and animals deficient in single ZO proteins [Umeda et al., 2004; Adachi et al., 2006] do not express different phenotypes. The different proteins can apparently replace each other, perhaps because of their sequential and functional identity within the hinge region.

In the hinge region, a coiled coil-helix (ZO-$1^{600-624}$) has been predicted [Mueller et al., 2005]. A coiled coil arises from the interaction between different helices. Thus, the hinge associates to the coiled coil-helices of occludin [Schmidt et al., 2004]. As far as known, the proteins tested in our study include helical segments and bind, at least, to the central part of the coiled coil-helix G^{606}LRSS KRNLR KSRE^{619}. This part is the segment which contains serines (ZO-$1^{609/610}$) which can be phosphorylated [Balda and Matter, 2000] and, hence, are of regulatory relevance. Consequently, the helix–helix interactions can be influenced by the phosphorylation of the hinge, as indicated by our binding experiments with Gα-proteins. Gα-proteins, on the opposite side, also contain helices known to associate with target proteins [Willard et al., 2008].

Under control conditions, the association of ZO-1 and G-proteins is weak in the cellular environment as we see weak colocalization. After G-protein activation, binding is intensified, becomes relevant to regulation and has physiological consequences. Now, colocalization at the cell membrane is clearly increased as supported by the significant increase in the colocalization coefficient. That means that G-protein activation is of regulatory relevance for ZO-1 and $G\alpha_{12}$ and includes a direct physical contact. As the activation of $G\alpha_{12}$ abolishes the TJ function [Meyer et al., 2002], one can assume that this pathway may impair the scaffolding function of ZO-1 for TJ. Phosphorylation in the hinge region, directly prior to the coiled coil-helix [Balda et al., 1996], may result in specific effects. For instance, the association of occludin to ZO-1 is reduced by the phosphorylation of ZO-1. In contrast, both Gα-proteins associate more strongly to the phosphorylation-mimicking mutations of ZO-1 than does the wild type.

Taken together, one can conclude that the hinge region of ZO-1 is a unique adaptor area, susceptible to a wide variety of proteins with a wide variety of cellular functions. Thus, the novel binding partners are potential targets for the design of new pharmacological approaches to influence cellular contacts specifically. ZO proteins connect these functions to various types of cell–cell contacts. These contacts may include, for example, TJ, adherens junctions, desmosomes and may control paracellular tightness, cell proliferation, cell–cell connections, or cell–cell communication. The elucidation of the interacting sequences and of the mode of interaction should improve our understanding of the molecular composition and regulation of cellular contacts.

ACKNOWLEDGMENTS

Authors thank Dr. S. Offermanns/Heidelberg, Germany for providing us with the plasmid encoding $G\alpha_{12}$, Dr. A. Lippoldt/Berlin, Germany for RGS5, and Dr. F. Hofmann for the β2-subunit. Support: DFG BL308/6-4, 7-4, and 9-1.

REFERENCES

Adachi M, Inoko A, Hata M, Furuse K, Umeda K, Itoh M, Tsukita S. 2006. Normal establishment of epithelial tight junctions in mice and cultured cells lacking expression of ZO-3, a tight-junction MAGUK protein. Mol Cell Biol 26:9003–9015.

Andreeva AY, Piontek J, Blasig IE, Utepbergenov DI. 2006. Assembly of tight junction is regulated by the antagonism of conventional and novel protein kinase C isoforms. Int J Biochem Cell Biol 38:222–233.

Balda MS, Anderson JM, Matter K. 1996. The SH3 domain of the tight junction protein ZO-1 binds to a serine protein kinase that phosphorylates a region C-terminal to this domain. FEBS Lett 399:326–332.

Balda MS, Matter K. 2000. The tight junction protein ZO-1 and an interacting transcription factor regulate ErbB-2 expression. EMBO J 19:2024–2033.

Bazzoni G, Martinez-Estrada O, Orsenigo F, Cordenosi M, Citi S, Dejana E. 2000. Interaction of junctional adhesion molecule with the tight junction components ZO-1, cingulin, and occludin. J Biol Chem 275:20520–20526.

Chen J, Pan L, Wei Z, Zhao Y, Zhang M. 2008. The membrane association domain of RGS16 contains unique amphipathic features that are conserved in RGS4 and RGS5. EMBO J 27:2113–2123.

Cocucci E, Racchetti G, Podini P, Rupnik M, Meldolesi J. 2004. Enlargeosome an exocytic vesicle resistant to nonionic detergents, undergoes endocytosis via a nonacidic route. Mol Biol Cell 15:5356–5368.

Costes SV, Daelemans D, Cho EH, Dobbin Z, Pavlakis G, Lockett S. 2004. Automatic and quantitative measurement of protein–protein colocalization in live cells Biophys J 86:3993–4003.

Denker BM, Saha C, Khawaja S, Nigam SK. 1996. Involvement of a heterotrimeric G protein α subunit in tight junction biogenesis. J Biol Chem 271:25750–25753.

Fanning AS, Jameson BJ, Jesaitis LA, Anderson LM. 1998. The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem 273:29745–29753.

Fanning AS, Ma TY, Anderson JM. 2002. Isolation and functional characterization of the actin-binding region in the tight junction protein ZO-1. FASEB J 16:1835–1837.

Fanning AS, Little BP, Rahner C, Utepbergenov D, Walther Z, Anderson JM. 2007. The unique-5 and -6 motifs of ZO-1 regulate tight junction strand localization and scaffolding properties. Mol Biol Cell 18:721–731.

Frank R, Overwin H. 1996. SPOT synthesis: Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. Methods Mol Biol 66:149–169.

Gaibelet G, Meilhoc E, Riond J, Saves I, Exner T, Liaubet L, Nurnberg B, Masson JM, Emorine LJ. 1999. Nonselective coupling of the human mu-opioid receptor to multiple inhibitory G-protein isoforms. Eur J Biochem 261:517–523.

Gentil BJ, Benaud C, Delphin C, Remy C, Berezowski V, Cecchelli R, Feraud O, Vittet D, Baudier J. 2005. Specific AHNAK expression in brain endothelial cells with barrier properties. J Cell Physiol 203:362–371.

Gohla A, Offermanns S, Wilkie TM, Schultz G. 1999. Differential involvement of G alpha(12) and G alpha(13) in receptor-mediated stress fiber formation. J Biol Chem 274:17901–17907.

Haase H, Podzuweit T, Lutsch G, Hohaus A, Kostka S, Lindschau C, Kott M, Kraft R, Morano I, 1999. Signaling from beta-adrenoceptor to L-type calcium channel: Identification of a novel cardiac protein kinase A target possessing similarities to AHNAK. FASEB J 13:2161–2172.

Haase H, Alvarez J, Petzhold D, Doller A, Behlke J, Erdmann J, Hetzer R, Regitz-Zagrosek V, Vassort G, Morano I. 2005. Ahnak is critical for cardiac Ca(V)1.2 calcium channel function and its beta-adrenergic regulation. FASEB J 19:1969–1977.

Haase H. 2007. Ahnak, a new player in beta-adrenergic regulation of the cardiac L-type Ca^{2+} channel Cardiovasc Res. 73:19–25.

Hohaus A, Person V, Behlke J, Schaper J, Morano I, Haase H. 2002. The carboxyl-terminal region of ahnak provides a link between cardiac L-type Ca^{2+} channels and the actin-based cytoskeleton. FASEB J 16:1205–1216.

Itoh M, Furuse M, Morita K, Kubota K, Saitou M, Tsukita S. 1999. Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2 and ZO-3, with the COOH termini of claudins. J Cell Biol 147:1351–1363.

Lippoldt A, Kirsch T, Rascher G, Kalbacher H, Wolburg H, Haller H. 2001. RGS5 is new a regulatory protein in tight junctions of endothelial cells is decreased in cerebral endothelial cells of SHRSP. Hypertension 38:964.

Lye MF, Fanning AS, Su Y, Anderson JM, Lavie A. 2010. Insights into regulated ligand binding sites from the structure of ZO-1 Src homology 3-guanylate kinase module. J Biol Chem 285:13907–13917.

Masuko N, Makino K, Kuwahara H, Fukunaga K, Sudo T, Araki N, Yamamoto H, Yamada Y, Miyamoto E, Saya H. 1999. Interaction of NE-dlg/SAP102, a neuronal and endocrine tissue-specific membrane-associated guanylate kinase protein, with calmodulin and PSD-95/SAP90—A possible regulatory role in molecular clustering at synaptic sites. J Biol Chem 274:5782–5790.

McGee AW, Dakoji SR, Olsen O, Bredt DS, Lim WA, Prehoda KE. 2001. Structure of the SH3-guanylate kinase module from PSD-95 suggests a mechanism for regulated assembly of MAGUK scaffolding proteins. Mol Cell 8:1291–1301.

Meyer TN, Schwesinger C, Denker BM. 2002. Zonula occludens-1 is a scaffolding protein for signaling molecules—G alpha(12) directly binds to the Src homology 3 domain and regulates paracellular permeability in epithelial cells. J Biol Chem 277:24855–24858.

Mueller SL, Portwich M, Schmidt A, Utepbergenov DI, Huber O, Blasig IE, Krause G. 2005. The tight junction protein occludin and the adherens junction protein α-catenin share a common interaction mechanism with ZO-1. J Biol Chem 280:3747–3756.

Nitta T, Hata M, Gotoh S, Seo Y, Sasaki H, Hashimoto N, Furuse M, Tsukita S. 2003. Size-selective loosening of the blood–brain barrier in claudin-5-deficient mice. J Cell Biol 161:653–660.

Pozgajova M, Sachs UJH, Hein L, Nieswandt B. 2006. Reduced thrombus stability in mice lacking the alpha(2A)-adrenergic receptor. Blood 108:510–514.

Rebres RA, Roach TI, Fraser ID, Philip F, Moon C, Lin KM, Liu J, Santat L, Cheadle L, Ross EM, Simon MI, Seaman WE. 2011. Synergistic Ca^{2+} responses by G{alpha}i- and G{alpha}q-coupled G-protein-coupled receptors require a single PLC{beta} isoform that is sensitive to both G{beta}{gamma} and G{alpha}q. J Biol Chem 286:942–951.

Saha C, Nigam SK, Denker BM. 1998. Involvement of G alpha(i2) in the maintenance and biogenesis of epithelial cell tight junctions. J Biol Chem 273:21629–21633.

Schmidt A, Utepbergenov DI, Krause G, Blasig IE. 2001. Use of surface plasmon resonance for real-time analysis of the interaction of ZO-1 and occludin. Biochem Biophys Res Commun 288:1194–1199.

Schmidt A, Utepbergenov DI, Mueller SL, Beyermann M, Schneider-Mergener J, Krause G, Blasig IE. 2004. Occludin binds to the SH3-hinge-GuK unit of zonula occludens protein 1—Potential mechanism of tight junction regulation. Cell Mol Life Sci 61:1354–1365.

Soma T, Chiba H, Kato-Mori Y, Wada T, Yamashita T, Kojima T, Sawada N. 2004. Thr207 of claudin-5 is involved in size-selective loosening of the endothelial barrier by cyclic AMP. Exp Cell Res 300:202–212.

Sussman J, Stokoe D, Ossina N, Shtivelman E. 2001. Protein kinase B phosphorylates AHNAK and regulates its subcellular localization. J Cell Biol 154:1019–1030.

Taliana L, Benezra M, Greenberg RS, Masur SK, Bernstein AM. 2005. ZO-1: Lamellipodial localization in a corneal fibroblast wound model. Invest Ophthalmol Vis Sci 4:96–103.

Umeda K, Matsui T, Nakayama M, Furuse K, Sasaki H, Furuse M, Tsukita S. 2004. Establishment and characterization of cultured epithelial cells lacking expression of ZO-1. J Biol Chem 27:44785–44794.

Utepbergenov DI, Fanning AS, Anderson JM. 2006. Dimerization of the scaffolding protein ZO-1 through the second PDZ domain. J Biol Chem 281:24671–24677.

Walter JK, Castro V, Voss M, Gast K, Rueckert C, Piontek J, Blasig IE. 2009. Redox-sensitivity of the dimerization of occludin. Cell Mol Life Sci 66:3655–3662.

Willard FS, Zheng Z, Guo J, Digby GJ, Kimple AJ, Conley JM, Johnston CA, Bosch D, Willard MD, Watts VJ, Lambert NA, Ikeda SR, Du Q, Siderovski DP. 2008. A point mutation to Gαi selectively blocks GoLoco motif binding: Direct evidence for Gα·GoLoco complexes in mitotic spindle dynamics. J Biol Chem 28:36698–36710.

Zhou J, Moroi K, Nishiyama M, Usui H, Seki N, Ishida J, Fukamizu A, Kimura S. 2001. Characterization of RGS5 in regulation of G protein-coupled receptor signaling. Life Sci 68:1457–1469.