The Tight Junction Protein Occludin and the Adherens Junction Protein α-Catenin Share a Common Interaction Mechanism with ZO-1*

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Received for publication, October 5, 2004, and in revised form, November 15, 2004. Published, JBC Papers in Press, November 16, 2004, DOI 10.1074/jbc.M411365200

The exact sites, structures, and molecular mechanisms of interaction between junction organizing zona occludens protein 1 (ZO-1) and the tight junction protein occludin or the adherens junction protein α-catenin are unknown. Binding studies by surface plasmon resonance spectroscopy and peptide mapping combined with comparative modeling utilizing crystal structures led for the first time to a molecular model revealing the binding of both occludin and α-catenin to the same binding site in ZO-1. Our data support a concept that ZO-1 successively associates with α-catenin at the adherens junction and occludin at the tight junction. Strong spatial evidence indicates that the occludin C-terminal coiled-coil domain dimerizes and interacts finally as a four-helix bundle with the identified structural motifs in ZO-1. The helix bundle of occludin1066–1291 and α-catenin159–906 interacts with the hinge region (ZO-1659–632 and ZO-1591–627, respectively) and with (ZO-1726–754 and ZO-1756–781) in the GuK domain of ZO-1 containing coiled-coil and α-helical structures, respectively. The selectivity of both protein-protein interactions is defined by complementary shapes and charges between the participating epitopes. In conclusion, a common molecular mechanism of forming an intermolecular helical bundle between the hinge region/GuK domain of ZO-1 and α-catenin and occludin is identified as a general molecular principle organizing the association of ZO-1 at adherens and tight junctions.

Different junctional complexes such as adherens junctions and, in specialized tissues, tight junctions, gap junctions, and desmosomes connect cells in multicellular organisms. TJ seal the most apical-lateral parts of cells and constitute a diffusion barrier for the paracellular flow of molecules and serve as a fence between the apical and basolateral membrane compartments (1). In contrast, AJ play important roles in cell adhesion, migration, morphogenesis, and proliferation. AJ represent Ca2+-dependent cell-cell contacts localized basolateral of TJ, where transmembrane proteins of the cadherin family mediate adhesion. Assembly of AJ is a prerequisite for the formation of TJ and desmosomes (2). β- and α-catenin bind to the cadherin cytoplasmic domain and link the cadherin-catenin complex to the F-actin cytoskeleton. An important scaffolding protein in cell-cell contacts is the zona occludens protein 1. ZO-1 is a membrane associated guanylate kinase homologue composed of the following domains: three PDZ (PSD95/Dlg/ZO-1), a SH3, a GuK (3), an actin binding region (4), and a ZU5 (ZO-1 and Unc5-like netrin receptor domain) according SMART (Simple Modular Architecture Research Tool (smart.embl-heidelberg.de) data base (5)). In non-epithelial cells ZO-1 is a major component of AJ, whereas in epithelial cells it is localized at TJ by directly binding to claudins (6). Occludin, one of the transmembrane proteins of TJ, is a multiphosphoprotein involved in regulation of TJ (7). It has four transmembrane domains with two extracellular loops and a cytosolic N and C terminus. A sequence of 244 amino acids in human ZO-1563–736 containing the GuK domain and an acidic region C terminus to GuK binds to the complete intracellular C-terminal tail of chicken occludin (8).

ZO-1 also binds to the AJ protein α-catenin (9) and to connexins in gap junctions (10), indicating a general scaffolding function of ZO-1 in junctional complexes. α-Catenin consists of several four-helix-bundle domains (vinculin homology domains, VHI–3) and binds β-catenin via an intermolecular helix bundle mechanisms within the E-cadherin-catenin complex at the intracellular side of AJ, where one helix of β-catenin interacts with one helix bundle (VH1) of α-catenin (11, 12). It was shown that the complete α-catenin sequence (13) and truncated mouse α-catenin569–906, α-catenin563–906 (9) constructs, bind the N-terminal half of ZO-11–562. Although several proteins are known that are involved in TJ and AJ, one reason for the poor understanding of their molecular interaction mechanism and regulation is the poor availability of structure-functional data for most of these proteins and their potential interactions. Moreover, the detailed association sites, three-dimensional structures, and molecular interaction mechanisms of the participating proteins are unknown.

In this report systematic studies on structure function relationships of junctional proteins are presented to discover the protein-protein interaction principles involved in cell communication. To provide support for the molecular understanding of the intracellular recruiting mechanisms at TJ and AJ, we determine here how the TJ protein occludin and the AJ protein α-catenin interact with the junctional recruiting protein ZO-1.

* This work was supported by Deutsche Forschungsgemeinschaft Grant DFG BL308/6. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ The abbreviations used are: TJ, tight junction(s); AJ, adherens junction(s); CC, coiled-coil; GGC, genetic computer group; GST, glutathione S-transferase; MAGUK, membrane associated guanylate kinase homologue; MBP, maltose binding protein; PDB, Protein Data Bank; SH3, Src homology domain 3; SPR, surface plasmon resonance spectroscopy; VHI–3, vinculin homology domains 1–3; ZO-1, zona occludens protein 1.
In detail, we wanted to identify determinants that are involved in the intermolecular interaction and to obtain indications for their spatial cooperation. Our strategy was built on our findings that due to high sequence homology, the C-terminal coiled-coil domain of occludin shares a common structure with four helical bundle fragments found in the crystal structure of α-catenin.

In this study we demonstrate the localization of coiled-coil motifs and α-helical elements in the C-terminal part of occludin at the C-terminal half of α-catenin and at the SH3-hinge-GuK unit of ZO-1 as intermolecular association sites and determine their relative spatial orientation. Moreover, our data provide strong support that α-catenin and occludin both interact in a four-helix-bundle motif with the same epitopes of ZO-1, namely with coiled-coil and α-helical elements of its SH3-hinge-GuK unit.

**MATERIALS AND METHODS**

**Biocomputing**—The GCG program package (GCG Wisconsin package Accelrys Inc., San Diego, CA) was used for sequence comparisons. If not mentioned otherwise the comparison matrix blossom62 was used to calculate the percentage of similarity. For secondary structure predictions www.compbio.dundee.ac.uk/~ww-pred was used, calculating a consensus prediction of the query sequence from several secondary structure prediction programs. COILS/scan of the GCG program package COILS (www.ch.embnet.org/software/COILS_form.html); based on the algorithm of Lupas (14, 15) and MATCH (cis.poly.edu/~jps) (16) were applied to identify helices with potential coiled-coil (CC) properties. COILS used MTIDK (a scoring matrix derived from myosins, tropomyosin, intermediate filaments, desmosomal proteins, and kinesins) as a training set for the CC prediction with an option to weigh the CC-heptad core position a and d 2.5 times more to exclude false positive predictions of charge-rich sequences. Except for the program MATCH, which aligns the sequence to the seven positions (a–g) of the coiled-coil heptad motif, the other two programs allow for a search of CC helices to adjust the search window to 14, 21, or 28 amino acids. If not mentioned otherwise, all sequence numbers were derived from mouse.

**Homology Modeling**—For selection of suitable structural templates, we first searched the PDB data base (17) for sequence similarity using FASTA, a program for fast alignment. The sequences of occludin (Swissprot accession number Q61146) and ZO-1 (P39447) were applied to the corresponding templates using the biopolymer module of the program Sybyl 6.8 from Tripos Inc. Small changes in loops were done by the loop search function of Sybyl, whereas large loops were modeled by assembling overlapping structural fragments comparable with the ROSETTA algorithm (18). The resulting models were minimized with an AMBER 4.1 force field and simulated with a 0.5-ns molecular dynamics run. The geometric accuracy was analyzed by using the PROCHECK program (19).

**Overexpression and Purification of Occludin, ZO-1, and α-Catenin**—Occludin constructs were produced by PCR from the complete C-termin- nal sequence (amino acids 264–521) as described (20). ZO-1 sequences containing the SH3 domain were derived from full-length cDNA of human ZO-1 (a kind gift from Alan Fanning, Chapel Hill, NC (8)). GuK sequences were generated from mouse ZO-1 cDNA by PCR (20). Full-length mouse α-catenin cDNA was isolated from pCIneo-α-catenin (21). Constructs of ZO-1 and occludin were cloned into the BamHI and SalI sites of the pGEX-4T-1 vector and transformed into Escherichia coli (TOP10F' Invitrogen). The expressed GST fusion proteins were purified on glutathione-agarose affinity columns (Sigma-Aldrich), washed with 20 mM Tris/Cl, pH 7.8, 200 mM NaCl, 1 mg/ml RNase, 1 mM proteinase inhibitor (Sigma-Aldrich), and eluted with 20 mM Tris/Cl, pH 7.8, and 2 mM glutathione. Subsequently, glutathione was removed by a HiPrep 26/10 desalting column (Amersham Bioscience).

**Surface Plasmon Resonance Spectroscopy; Protein-Protein Binding Studies**—A Biacoore 2000 instrument and CM5 chips (BIACORE AB, Uppsala, Sweden) were used as reported (20). Briefly, immobilization of MBP fusion proteins was performed according to the manufacturer's instructions using 10 μM glutathione buffer, pH 4. To adjust protein densities on the chip, an occludin construct containing all predicted secondary structural features (occludin406–521) was always immobilized as internal control to be compared with other fragments used for binding studies with ZO-1 constructs. The binding procedure for all experiments was 4 min of injection of sample at a flow rate of 8 μl/min using running buffer (20 mM Tris/Cl, pH 7.8, 200 mM NaCl, 5 mM maltose). Peptide Epitope Mapping Analysis—To analyze occludin and α-catenin epitopes, peptide spots with peptides of 29 amino acids in length spanning occludin406–521 with a shift of 1 amino acid between each peptide were synthesized on Whatman 50 paper (Medstone, England) (22). Peptides from ZO-1 containing the CC1 (ZO-1597–635) and H2 regions (ZO-1745–772) were synthesized with an N-terminal linker of two β- alanines and one cysteine and coupled to the red dye tetramethylrhodamine-6-maleimide (Molecular Probes Europe BV, Leiden, The Netherlands). The membranes were incubated with the labeled peptides of ZO-1 and after washing with phosphate-buffered saline, the interacting peptides were directly visible (23). The dye coupled to mercaptopropanol was used as a negative control.

**RESULTS**

Coiled-coil Helical Regions, as a Structural Interaction Motif, Occur in Occludin, α-Catenin, and in ZO-1

Bioinformatic studies identified coiled-coil helical patterns in occludin, α-catenin, and ZO-1. The C-terminal part of the occludin cytoplasmic tail (mouse amino acids 406–521) was predicted to consist of multiple helices termed Oc-Ha, Oc-Hb, Oc-Ca, Oc-Cb, and Oc-Hc (occludin406–414, 422–433, 438–463, 471–488, 494–514, Fig. 1A, Table I). Some helices (Oc-Ca, Oc-Cb, and partly Oc-Hb) exhibited potential coiled-coil properties with 100% probability. The classical coiled-coil (CC) pattern shows a heptad pattern of amino acid (abcdefg) with the positions a and d mostly hydrophobic and forming the core of two or more interwoven helices (24). Comparison of the available crystal structures for α-catenin domains and the homologous domains in vinculin (PDB entries see Fig. 1B) indicates common four-helix-bundle structure motifs at VH1–3, which also contain portions of coiled-coil properties (Table I). In ZO-1, two relatively short coiled-coil helices were predicted as potential protein-protein interaction regions; that is, in the hinge region between the SH3 and GuK domains (mouse ZO-1511–624) termed ZO-1-CC1 and within the C-terminal sequence of the GuK domain (ZO-1784–799) termed ZO-1-CC2 (Fig. 1C). ZO-1-CC1 is located in the N terminus before the amino acids of the reported site to interact with occludin (8). Coiled-coil patterns for CC1 were predicted with significant probability only if the smallest search window (size 14) of the program COILS was applied. The predicted coiled-coil probability (60%) was not affected by using the weighting function of COILS (61%) showing the influence of the hydrophobic heptad positions a and d. This confirms a significant propensity to form a short coiled-coil helix in ZO-1-CC1.
Sequence-Structure-Function Relationships of Occludin, α-Catenin, and ZO-1

To provide further support for the predictions reported above, homologous molecular models were generated based on structural templates from the protein structure data base (PDB).

Occludin—Two homologous structural fragments identified by sequence similarity in known crystal structures confirmed the predicted secondary structure including coiled-coil elements in the C-terminal portion of occludin, accounting for a helix bundle fold. Surprisingly, the closest structural homology was found in a part of VH1 domain of α-catenin, revealing a four-helix-bundle (PDB code 1DOV). Occludin436–521 (containing the structural elements Oc-CCa/b, -Hc) showed 37% sequence similarity to the α-catenin portion 1DOV172–262 (Fig. 1, A and B). In the N terminus (occludin406–435 corresponding to

Fig. 1. Sequences of occludin, α-catenin, and ZO-1, aligned with sequences of crystal structures used as structural templates for generating structural homologous models. Sequence similarities are highlighted according to pam250 matrix for identity (black), high (dark gray), and low (light gray) similarity. White tubes, α-helix; arrows, β-strands; solid line, derived from template; dashed line, predicted; gray tube, predicted coiled-coil helix. A, mouse occludin400–521 with template fragments; PDB entries 1BF5 (STAT-1272–306) and 1DOV (α-catenin179–262), B, sequence of mouse α-catenin containing three VH regions (28) compared with crystal structures of α-catenin and vinculin with PDB entries and sequence numbers as indicated (compressed presentation). Four helix bundles are common to all vinculin homologous regions. C, mouse ZO-1518–812 and crystal structure of SH3-hinge-GuK unit of PSD-95 (PDB code 1KJW). High sequence similarity exists in SH3 and GuK but not in hinge and GMP binding loop; the hinge is larger and contains a predicted coiled-coil helix CC1; the GMP binding loop of ZO-1-GuK is shorter and contains no GMP binding pattern.
Oc-Ha, -Hb), the model was extended by a template from the coiled-coil from four-helix-bundle helix protein STAT-1 comprising a negatively charged helix-turn-helix region (PDB code 1BP5 787–806; 55% similarity, Fig. 1A). Because the coiled-coil prediction includes Oc-CCa and nearly the complete Oc-Hb, we postulate a continuous helix in this region, and the fragments were connected accordingly. Our model clearly indicated that occludin very likely adopts a helical bundle fold similar to α-catenin (see below). However, we cannot exclude that the helix Oc-Hc with mainly basic residues could flip toward the acidic helix Oc-Ha, resulting in three helix bundles (Fig. 2C). The electrostatic potential at the surface of the occludin monomer (Fig. 2D) exhibited negative potentials at the two ends of the bundle interrupted by a positive potential caused by the bend basic α-helix Oc-Hc (Fig. 2C).

α-Catenin—Four structures from α-catenin are available comprising the vinculin homologous regions VH1 and VH2, each consisting of four-helix bundles partly containing coiled-coil properties. VH3 of α-catenin can be represented by vinculin structures (Fig. 1B). VH2 consists of two four-helix bundles starting at residues 377 and 509, respectively. The electrostatic potential at the molecular surface for the structure of α-catenin 505–630 (1H6C) shows occludin-like monomers at both ends of the four-helix-bundle negatively charged areas (Fig. 2A). The negative electrostatic potential on the surface of α-catenin 685–925 corresponding to VH3 (model based on vinculin, 1RKE) is even extended to a long continuous acidic patch on one side (Fig. 2B).

ZO-1—For homology modeling of the GuK domain, guanylate kinase structures of the yeast enzyme (PDB codes 1GKY, 1EX6, 1EX7) and from the MAGUK protein human CASK (PDB code 1KGd) were available. The most impact to our structure-function considerations had structures containing the SH3 and GuK domain of another MAGUK protein, PSD-95 (PDB codes 1KJW, 1JXM, 1JXO). Therein SH3 and GuK were linked to form a common structural unit stabilized via the interaction of six β-strands (A–F). The β-strands were located inside the SH3 domain (A–D) and N-terminal (E) and C-terminal (F) to GuK (25, 26) (Fig. 1C and 2D). The high sequence similarity (45%) when between ZO-1 1518–1806 and PSD-95 1430–724 is even more pronounced when only the SH3 (51%) or GuK (45%) domains were compared (Fig. 1C). Only the hinge region between the SH3 and GuK and the GMP binding loop within GuK of PSD-95 showed differences in length and sequence compared with ZO-1. The hinge region in ZO-1 is about 20 residues longer than in PSD-95, and additionally it contains the predicted ZO-1-CC1, whereas the GMP binding loop in ZO-1 is 30 amino acids shorter (Fig. 1C). Alignment of sequences from more than 30 MAGUK proteins (not shown) revealed that the SH3-hinge-GuK unit seems to be a common feature of this protein family. In the homology model for ZO-1 we modified the hinge region at two sites; first, by elongation of the template helix after the C terminus of the SH3 domain according α-helix predictions (with PDB code 1EPU 170–181); second, because the hinge region of ZO-1 is longer than in the template used (PSD95), the predicted coiled-coil region ZO-1-CC1 (structural fragment adopted from the template PDB 1A93 17–45) was inserted into the ZO-1 hinge region. The resulting coiled-coil helix CC1 (red/blue) at the hinge region (red) and the α-helices H1 and H2 (blue) from GuK contain a surplus of positive charges and are located in spatial proximity at the surface of SH3-hinge-GuK unit (Figs. 1 and 2F). At the molecular surface of the ZO-1-SH3-hinge-GuK unit they form a large area of positive electrostatic potential (Fig. 2E).

### Table I

Comparison of coiled-coil sequence regions in occludin, α-catenin, and ZO-1 from mouse predicted by different methods (MATCH, COILS, and Coliscan)

| Protein-substructural element (see Fig.1) | Predicted region | CC probability |
|------------------------------------------|------------------|----------------|
|                                          | MATCH | COILS (window size) | COILS (MTIDK) |
|                                          |       |                   | Normal | Weighted |
| ZO-1-CC1                                 | 607–624 | 611–624 (w14) | 50 60 | 61 |
| ZO-1-CC2                                 | 785–797 | 784–798 (w14) | 92 94 | 91 |
| Oc-Hb, Oc-CCa-Oc-CCb                      | 426–498 | 424–488 (w28) | 100 | 100 |
| α-Catenin -VH2                           | 328–385 | 327–351 (w21) | 51 55 | 28 |
| α-Catenin N terminus of VH3              | 667–702 | 675–707 (w28) | 99 99 | 69 |
| α-Catenin C terminus of VH3              | 873–889 | 876–889 (w14) | 52 | 61 |

* No significant prediction.

b Scoring matrix derived from myosins, tropomyosins, intermediate filaments, desmosomal proteins, and kinesins (14).

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Occludin and α-Catenin Interact with ZO-1 via the Same Epitopes

To prove the hypothesis of a common interaction mechanism between occludin and ZO-1 and α-catenin and ZO-1, we analyzed constructs that were systematically varied according to the secondary structural elements in binding studies using surface plasmon resonance ( SPR). Occludin 406–521 comprising the predicted coiled-coil domain, exhibited one pure band after purification and SDS-PAGE, suggesting a stable/correctly folded structure. Deletion mutants of occludin 406–521 comprising Oc-Ha, Oc-Hb, Oc-CCa, Oc-CCb, and Oc-He bound to ZO-1 1505–724 (Fig. 3A) and other ZO-1 fragments (Fig. 3B). However, N-terminal extension of occludin coiled-coil domain lacking structure predictions (occludin 378–405) strongly decreased the ZO-1 binding, probably by masking the binding site. We observed also that the complete C-terminal tail occludin 264–521 was unstable. N-terminal deletion of Oc-Ha and Oc-Hb from occludin coiled-coil domain led to decreased binding. Truncation of Oc-He at the C-terminal side led to an increase of binding to ZO-1-GuK fragments. Further C-terminal deletion indicated that the essential binding region is defined by occludin 406–480 comprising Oc-Ha, Oc-Hb, Oc-CCa, and Oc-CCb (Fig. 3A). Comparison of ZO-1 constructs spanning different regions around GuK emphasized the importance of the ZO-1 features CC1 in the hinge region and of H1 and H2 in GuK for the association with occludin. C-terminal truncations excluding ZO-1-CC2 from GuK increased the binding to occludin. A possible reason for this is that the acidic residues after β-strand F in the C-terminal tail of GuK may mask the basic patch of GuK for interaction (Fig. 1C). The maximum binding intensity to all occludin constructs was obtained with ZO-1 589–722 (Fig. 3B). To test binding of ZO-1 to α-catenin we used α-catenin 508 and catenin 509–906 corresponding to VH1.
plus the first portion of VH2 and the second four-helix bundle of VH2 plus VH3, respectively. In addition, α-catenin\(^{631-766}\) a truncated construct with a CC prediction and similarity to occludin, was also tested (Fig. 3B). But the best binding was observed with the larger construct α-catenin\(^{509-906}\). Less ZO-1 binding for the truncated construct α-catenin\(^{631-766}\) indicates that not the CC-region alone, but the complete helix bundle structure of VH3, is necessary for the interaction. ZO-1 binding to α-catenin\(^{1-508}\) (VH1, first half VH2, VH3 region) was weaker than to α-catenin\(^{509-906}\) (second half VH2, VH3 region) that corresponds to the findings that within VH1 contains no significant CC probabilities were predicted. The order of binding strength of the analyzed ZO-1 fragments to α-catenin was identical to their binding toward occludin (Fig. 3B). α-Catenin\(^{509-906}\) showed strong binding to ZO-1 and contains a coiled-coil prediction (amino acids 678–707) with sequence similarity (45%) to parts of Oc-CCa and Oc-Cb and bend basic helix Hc) and of STAT-1 (PDB code 1BF5; orange with helices Ha (surplus of acidic residues) and Hb; for details, see Fig. 1). Narrow yellow stripe, CC prediction. D, the electrostatic potentials at the surface of occludin show two negative potentials (red Ha; CCa/b) at both ends interrupted by a positive potential (blue Hc). E, large positive potential (blue) on SH3-hinge-GuK is caused by CC1, H1, and H2. F, ZO-1\(^{1318-806}\) model (backbone representation) based on the SH3-hinge-GuK unit structure of PSD-95 (PDB code 1KJW) with SH3 (yellow) and GuK (gray) linked by the hinge region (red). CC1 (red/blue) of hinge region and helices H1 and H2 (blue) from GuK bound occludin coiled-coil domain are shown. The electrostatic potentials of monomeric occludin domain (D) and monomeric ZO-1 SH3-hinge-GuK unit (E) show no complementary patterns fitting to a 1:1 intermolecular interaction.

**Peptide Mapping; Occludin and α-Catenin Bind to the Same Peptide Epitopes in ZO-1**

To examine the binding epitopes of the interacting ZO-1 elements CC1 and H2 in more detail, peptide mapping with systematically varied sequences of occludin\(^{506-521}\) and α-catenin\(^{509-906}\) was performed. The two ZO-1 elements showed different and overlapping binding epitopes toward occludin peptides, whereas toward α-catenin the same interaction patterns were observed (Fig. 4). CC1 and H2 bound to spots 1–5 comprising occludin\(^{400-433}\), which corresponds to the features Oc-Ha/Hb. CC1 bound best to the occludin spots 81–93, corresponding to occludin\(^{400-433}\) (Fig. 4), which contained Oc-Hc. It also bound to spots 53–58, representing occludin\(^{452-485}\), a part of the coiled-coil domain that includes the turn between Oc-CCa and Oc-CCb and contains a surplus of acidic residues (Fig. 1A). H2 bound best to spots 46–60, spanning occludin\(^{445-487}\) (Fig. 3B). These data indicated that the ZO-1 peptides CC1 and H2 interact with coiled-coil epitopes Oc-CCa/CCb and α-helices Oc-Ha/Hb of the occludin coiled-coil domain. The interaction with α-catenin spot membrane showed for the two ZO-1 peptides CC1 and H2 nearly identical results. The recognized regions in α-catenin correspond to amino acids 509–541, 535–576, and 584–664 in the VH2 region that correspond to helices known from the crystal structures (Fig. 4). Moreover, the identified binding epitope α-catenin\(^{684-734}\) the coiled-coil prediction at α-catenin\(^{678-707}\) in VH3 (Table I). Most of the recognized peptide spots of α-catenin may carry a negative net charge, whereas the ZO-1 peptides are positively charged. In reciprocal experiments ZO-1 peptides
from hinge and GuK were spotted, and the binding of GST-
occludin$^{406-521}$ and GST-$\alpha$-catenin$^{509-906}$ was analyzed by chemiluminescence detection via anti-GST- horseradish peroxidase antibodies. Both protein constructs showed binding to the ZO-1$^{591-632}$, ZO-1$^{726-754}$, and ZO-1$^{756-781}$ corresponding to the ZO-1 features CC1, H1, and H2. Compared with occludin, the binding of GST-$\alpha$-catenin$^{509-906}$ generally seems to be weaker, and in ZO-1-CC1 only a shorter region, ZO-1$^{591-622}$ (for occludin ZO-1$^{591-632}$), was found as binding epitope (Fig. 5). All spotted peptides containing a cysteine such as Ha and Hc in occludin, were also tested in parallel experiments with serine instead of cysteine, revealing the same results. This indicates that disulfide bridges do not influence the results. Additionally synthesized peptide spots with glutamate at the serine residues Ser-609 and Ser-610 to order to simulate a predicted phosphorylation indicate a decrease in occludin binding (not shown).
FIG. 4. Binding of tetramethylrhodamine-labeled ZO-1597–633 and ZO-1745–772 to peptide spot membranes of occludin400–521 and α-catenin509–906. ZO-1597–633 and ZO-1745–772 include the predicted coiled-coil helix CC1 of hinge region and the helical portion H2 of GuK. Each spot contains a 29-mer peptide shifted by one amino acid from one spot to the next spot (e.g., spots 1, occludin400–428 or α-catenin509–537; spot 2, occludin429–458 or α-catenin538–567, respectively). Boxed, consecutive peptides of high binding affinity. Note that CC1 and H2 bind to the same spots of α-catenin, indicating similar binding properties, whereas, binding to occludin differs regarding Hc of occludin (spots 81–94).

FIG. 5. Mapping of the occludin and α-catenin binding site in ZO-1. Binding of GST fusion proteins of occludin400–521 and α-catenin509–906 to peptide spot membranes representing the hinge region (ZO-1571–645) and second half of GuK domain (ZO-1726–810) was detected by chemiluminescence with horseradish peroxidase-labeled anti-GST antibodies. Each spot represents a 20-mer peptide shifted by one amino acid at the next spot (e.g., 1a, ZO-1571–590; 2a, ZO-1572–591). Dark spots represent binding. The occludin and α-catenin constructs bind to the same ZO-1 peptides comprising CC1, H1, and H2, whereas α-catenin binds weaker and to a shorter region of ZO-1-CC1.
**Interaction Patterns Suggest a Homodimer Model for Ocludin and SH3-Hinge-GuK Unit**

The electrostatic potentials of α-catenin<sup>509–630</sup> (Fig. 2A), the monomeric occludin (Fig. 2D), and the monomeric SH3-hinge-GuK unit (Fig. 2E) of ZO-1 had no sufficient complementary patterns in size and properties, which would fit (negative to positive) for a 1:1 intermolecular interactions. Only α-catenin<sup>685–805</sup> (VH3) and SH3-hinge-GuK unit of ZO-1 show complementary properties at their surfaces (Fig. 2, B and E). However, it is suggested that the respective parts (Oc-CCa/Oc-CCb and α-helices Oc-Ha/-Hb) of occludin may form a homodimer (27). Preliminary experimental data (mass spectrometry, size exclusion chromatography, light scattering) support this hypothesis. Accordingly, we generated a homodimer model of occludin coiled-coil domain. For construction of a dimer model, we flipped the basic helix Oc-Hc toward the acidic Oc-Ha helix, as described for an extra helix at α-catenin that flipped upon β-catenin binding (11).

Thus, we received two long helices associated to a four-helix-dimer bundle when using the crystal structure template (PDB code 1QU7) (Fig. 6A). In the resulting antiparallel dimer model of the occludin C-terminal coiled-coil domain, the negatively charged helix-turn-helix regions cause large patches with negative electrostatic potential at both ends of the helix bundle. These patches are connected by negative charges contributed by Oc-CCa forming a large contiguous negative electrostatic potential at one side of the surface (Fig. 6B). The considered cytoplasmic coiled-coil domain of occludin can dimerize in an anti-parallel manner just beneath the plasma membrane, because only the C-terminal half of the C-terminal tail is involved in the interaction, whereas more than 100 very likely unstructured amino acids act as a spacer. Moreover, this model is not only very similar to the four-helix-bundle crystal structure of α-catenin (28), but it also forms a comparable contiguous negative electrostatic potential as α-catenin<sup>685–805</sup> (Fig. 2B). Moreover, the linker region between VH2 and VH3 (no structure available) consisting of a surplus of acidic residues very like enhances the negative electrostatic potential of the measured α-catenin construct. Peptides corresponding to this linker region bind the basic ZO-1 peptide CC1 and H2 (Fig. 4).

To generate a dimer model of the ZO-1 SH3-hinge-GuK unit, we assembled two monomers in a way that the two positive patches of the electrostatic potential from the two monomers form a long contiguous positive charged area, whereas the interacting shapes of the two monomers fit very well to each other. Shapes and patterns of the electrostatic potential from this dimer model complementsarily match exactly with those of the occludin dimer (Fig. 6, B and C). This ZO-1 model is consistent with a possible formation of an open oligomer formed by a swapped hinge region as previously suggested (25).

Taken together, we identified specific sequence regions with α-helical and coiled-coil-character as common binding epitopes between the Tj-proteins ZO-1 and occludin and between ZO-1 and the AJ protein α-catenin as well. Our structure-function
study clearly indicates that this particular protein-protein interaction is caused by the complementary shapes and properties between the epitopes of the SH3-hinge-GuK region (CC1, H2; positively charged) in ZO-1 and the C-terminal coiled-coil domain (Oc-Ha, Oc-Hb, Oc-CCa, Oc-CCb; negatively charged) as a dimer in occludin and a negatively charged helical bundle (partially coiled-coil) in α-catenin as well.

**DISCUSSION**

Combining bioinformatic studies (sequence conservation information, comparative modeling) and different experimental binding techniques (SPR, peptide mapping) we identified key epitopes in occludin and α-catenin, interacting with ZO-1 via a common molecular mechanism. For the first time we present that the hinge-GuK region of ZO-1, which revealed maximum binding to occludin, also provides the best site to interact with the VH3 helical bundle segment in α-catenin. For all interacting epitopes known in the studied proteins, crystal structures of highly homologous sequences were identified (11) that show helical bundle and/or CC-properties as potential protein interaction motifs. We provide strong spatial indications by comparative models that the occludin C-terminal CC-domain interacts as a dimerized four-helix bundle and that α-catenin four-helix bundle structures interact in a similar way with ZO-1.

First we analyzed whether complementary structural interaction features exist in junctional proteins. Bioinformatic studies result in helical CC motifs (24) in the second half of cytosolic C-terminal part of occludin, in α-catenin, and around the ZO-1-GuK domain. The best structural template with closest sequence similarity to the occludin C-terminal CC-domain was a structure in α-catenin. Common to all three VH regions of α-catenin is a helix bundle structure according to the known crystal structures (PDB codes see Fig 1B; Ref. 28). Occludin and α-catenin obviously share the same structural properties; that is CC motifs and similar electrostatic properties within four-helix bundles. Because CC regions also have been predicted for hinge and GuK of ZO-1, a common interaction mechanism of occludin and α-catenin toward ZO-1 is hypothesized. Comparative modeling provides strong indications for a similar helix bundle interaction mechanism mediating the association between ZO-1 and occludin as well as ZO-1 and α-catenin, experimentally confirmed by binding studies using SPR and peptide mapping.

One of the two essential binding epitopes identified in ZO-1 includes the α-helices H1 and H2 located at the surface of the SH3-hinge-GuK structural model. This is consistent with previous observations that the GuK domain is involved in occludin binding (8). Occludin splice variants, where the C terminus is located extracellular instead of intracellular due to a lack of transmembrane domain four, were not localized at TJ (30). Occludin mutants without the intracellular C terminus were shown to disrupt TJ (31). These observations suggest that ZO-1 might organize occludin via its C terminus at TJ since in the absence of ZO-1 occludin is diffusely distributed in membranes (32). In addition, we identified the CC-motif ZO-1-CC1 right in the hinge between SH3 and GuK, as a second epitope involved in binding of ZO-1 to occludin and α-catenin.

The relevance of the ZO-1 sites is confirmed by independent methods. SPR binding studies of protein fragments reveal the importance of ZO-1-CC1 and -H1/H2 for binding. The different fragments exhibit similar strength of binding to occludin and α-catenin, whereas the complete SH3-hinge-GuK unit (ZO-1\(^{590-630}\)) showed no binding to α-catenin\(^{330-508}\) but association to occludin\(^{590-630}\) and α-catenin\(^{509-906}\). This agrees with earlier studies observing no binding of the first half of α-catenin to ZO-1 (9, 13). Moreover, we narrowed down the ZO-1 binding region of α-catenin\(^{509-906}\) to ZO-1\(^{589-772}\).

Using a second independent technique, peptide mapping, we defined binding sites for occludin\(^{406-521}\) and α-catenin\(^{509-906}\) in CC1, and H1 and H2 from ZO-1 (ZO-1\(^{591-632}\), \(^{726-754}\), and \(^{756-781}\)).

Peptide studies may result in artificial structures because an isolated peptide may not adopt the same conformation as in the native protein. However, the reciprocal approach with immobilized occludin and α-catenin peptides incubated with CC1 and H2 peptides of ZO-1 also showed results fitting to our interaction models. Here epitopes in occludin (Oc-Ha, -Hb, -CCa, -CCb) and in α-catenin (VH2, predicted CC-region in VH3) were detected. The three identified binding helices in ZO-1 have a surplus of basic residues, and the essential helices in occludin and α-catenin present a complementary surplus of acidic residues. This further supports the hypothesis that the hinge region and the GuK domain of ZO-1 interacts with both helix bundle domains in occludin and in α-catenin.

The importance of the hinge region between SH3 and GuK domains in MAGUK proteins is demonstrated by a deletion mutant of ZO-1, lacking the CC1-helix and the GuK domain (615–812), which remains intracellularly (33). Specific splice variants of MAGUK hDlg in this region lead to membrane or nuclear targeting (34). Interestingly in the hinge of ZO-1 a phosphorylation site was identified (35), suggesting regulatory relevance. This site is located at the predicted basic patch of our SH3-hinge-GuK model. A phosphorylation here would probably decrease the binding due to electrostatic repulsion. This is consistent with our peptide spotting results where these serines were substituted by glutamates.

Binding strength of ZO-1 to occludin/α-catenin is in a similar order of magnitude. This means for physiological conditions that α-catenin in a α-catenin-ZO-1 complex at AJ can be displaced by occludin. This assumption is supported by our finding that occludin binds stronger to the complete SH3-hinge-GUK unit as compared with α-catenin. Additionally, competition experiments show the same relative decrease of soluble occludin for the withdrawal of ZO-1 from α-catenin and occludin indicating an overlap of binding epitopes. During initial cell-cell contact formation, ZO-1 appears first to colocalize with E-cadherin. Later on, occluding-ZO-1 complexes are sorted out from E-cadherin to form TJ and AJ (29). Consequently, it is possible that ZO-1 first interacts with α-catenin in the E-cadherin-catenin complex and then with occludin at TJ via the same binding site.

The monomeric occludin model shows an elongated 3-helix bundle with negative electrostatic potentials at both termini formed by Oc-Ha and the Oc-CCa/CCb turn. The basic Oc-Hc located between these patches is not involved in the association (Figs. 2, C and D, and 3A). This agrees with overexpression studies of chicken occludin in Xenopus embryos where a deletion corresponding to Oc-Hc was tolerated, but further C-terminal truncations resulted in leaky phenotypes (36). The electrostatic potential of α-catenin\(^{509-630}\) structure shows a similar pattern as an occludin monomer (Fig. 2, A and D). In the monomeric SH3-hinge-GuK model, a distinct continuous basic patch is established by the ZO-1 elements H1/H2 of GuK and CC1 of the hinge (Fig. 2, E and F). Electrostatic potentials of α-catenin\(^{509-630}\) (VH2) and occludin\(^{506-521}\) compared with that of ZO-1\(^{518-806}\) suggest insufficient complementary properties and contact surfaces for optimal interaction. However, it has been shown that human occludin\(^{440-469}\) (consisting of Oc-CCa) is able to bind occludin from human T84 cells, suggesting dimerization (27). We therefore, developed an antiparallel homodimer model of occludin (Fig. 6, A and B), where CC-helix/turn/CC-helix motifs of two occludin molecules are twisted together to form a four-helix CC-bundle. This dimer model with
its long continuous negative electrostatic patch at one side possesses perfect size and complementary properties to associate with two SH3-hinge-GuK units of ZO-1. The negative electrostatic potential of α-catenin \(^{585–585}\) model (VH3) shows similarities to the occludin dimer patterns (Fig. 2B and 6B).

According to our dimer models we suggest a scenario where the four-helix bundle of an occludin dimer (or α-catenin four-helix bundle of VH regions) interacts with a ZO-1 dimer. The long continuous acidic patches of occludin find as counterpart large contiguous basic patches from two SH3-hinge-GuK units formed by ZO-1-CC1, -H1/-H2, and ZO-1-CC1’, -H1’/-H2’ (Fig. 6D). Upon adaptor binding, swapping of SH3 and GuK via the hinge region has been suggested for the MAGUK protein PSD95 to form a dimerized SH3-hinge-GuK interaction (25). Our SH3-hinge-GuK dimer model of ZO-1 allows such a swapping, likely caused by occludin acting as an adaptor. Although GuK remains in the same relative location, its SH3 domain can be swapped by minor conformational changes toward SH3’. Subsequently, GuK’ also remain in the same positions, but SH3’ would be swapped toward a SH3‘’ location allowing a linear oligomerization. This is supported by our findings of a direct binding of SH3-hinge-GuK unit of ZO-1 with itself in SPR experiments (Fig. 6E).

This scenario is also relevant for other MAGUK proteins where hinge regions play a functional role for oligomerization by domain swapping upon binding of adaptor molecules. Mutational and structural analyses of the joint closed structural unit of the SH3-GuK crystal structure of PSD95 favor this adaptor mechanism (25). The sequential domain order within MAGUKs is highly conserved (3). Similar SH3-hinge-GuK units, therefore, are postulated for other MAGUKs such as ZO-1. The hinge region considerably varies among MAGUKs in sequence and length and is suggested to represent the binding site of adaptor proteins (37–39).

Proteolysis experiments leading to a stable product consisting of human occludin \(^{404–522}\) fairly corresponds to our ZO-1 binding CC-domain of mouse occludin \(^{406–521}\). Binding data of human occludin \(^{404–522}\) to ZO-2 gave hints that two or more ZO-2 molecules interact with occludin (40) and, therefore, support our dimer model. Occludin knock out studies in mice show no direct changes in TJ-morphology but show a complex phenotype with postnatal growth retardation and abnormalities in several tissues and in sexual behavior (41). This indicates a possible role of occludin in regulatory processes via the here described adaptor mechanism.

Comparing our and literature data, we suggest that occludin and α-catenin, like adaptor proteins in other MAGUK proteins, trigger the ZO-1 hinge region to an “open” conformation. The open state results in domain swapping of the SH3 and GuK domain and, subsequently, in a linear dimerization or oligomerization as suggested for the MAGUK protein PSD95 (25). The fact that there may occur a linear oligomerization of ZO-1 when bound to occludin raises the idea that TJ strands may be organized by linear oligomerization of TJ associated proteins. Also AJ are organized along apical part of lateral cell membrane. The helical bundle interaction mechanism found in this paper is obviously common for protein-protein interactions between occludin/ZO-1 at TJ and ZO-1/α-catenin and α-catenin/β-catenin at AJ.

Acknowledgments—We thank Dr. A. S. Fanning for providing the ZO-1 plasmid as well as Gislinde Hartmann and Barbara Pisarska for technical assistance.

REFERENCES

1. Anderson, J. M. (2001) *News Physiol. Sci.* 16, 126–130
2. Gumbiner, B., Stevenson, B., and Grimaldi, A. (1988) *J. Cell Biol.* 107, 1575–1587
3. González-Mariscal, L., Betanzas, A., Nava, P., and Jaramillo, B. E. (2003) *Biophys. Mol. Biol.* 81, 1–14
4. Fanning, A. S., Ma, T. Y., and Anderson, J. M. (2002) *FASEB J.* 16, 1835–1837
5. Schultz, J., Milpetz, F., Bork, P., and Ponting, C. P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 5857–5864
6. Nagafuchi, A. (2001) *Curr. Opin. Cell Biol.* 13, 600–603
7. Andreeva, A. Y., Krause, E., Muller, E. C., Blasig, I. E., and Utebergorenberg, D. I. (2001) *J. Biol. Chem.* 276, 38480–38486
8. Fanning, A. S., Jameson, B. J., Jesaitis, I. A., and Anderson, J. M. (1998) *J. Biol. Chem.* 273, 29745–29753
9. Imamura, Y., Itoh, M., Maeno, Y., Tsukita, S., and Nagafuchi, A. (1999) *J. Cell Biol.* 144, 1311–1322
10. Kaulaulya, P., Reichert, M., and Hunziker, W. (2001) *FEBS Lett.* 505, 92–96
11. Pokutta, S., and Weis, W. (2000) *Mol. Cell* 5, 533–543
12. Vasioukhin, V., and Fuchs, E. (2001) *Curr. Opin. Cell Biol.* 13, 76–84
13. Itoh, M., Nagafuchi, A., Mersi, S., and Tsukita, S. (1997) *J. Cell Biol.* 138, 181–192
14. Lupas, A., Van Dyke, M., and Stock, J. (1996) *Science* 275, 1162–1164
15. Lupas, A. (1996) *Methods Enzymol.* 266, 513–525
16. Fischetti, V. A., Landau, G. M., Schmidt, J. P., and Sellers, P. (1993) *Information Processing Letters* 45, 11–18
17. Heyman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Ibañez, W. M., Weissig, H., Shindaylov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res.* 28, 235–242
18. Simons, K. T., Bonneau, R., Ruzyczki, I., and Baker, D. (1999) *Proteins, Suppl.* 3, 171–176
19. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) *J. Appl. Crystallogr.* 26, 283–291
20. Schmidt, A., Utebergorenberg, D. I., Krause, E., and Blasig, I. E. (2001) *Biochem. Biophys. Res. Commun.* 288, 1194–1199
21. Huber, O., Krohn, M., and Kemler, R. (1997) *J. Cell Sci.* 110, 1759–1765
22. Kramer, A., and Schneider-Mergener, J. (1998) *Methods Mol. Biol.* 87, 25–39
23. Toepfer, P., Krause, T., Guffler, S., Fries, J. R., Matzdorf, T., Oechslin, H., and Schneider-Mergener, J. (2003) *Angew. Chem. Int. Ed. Engl.* 42, 1136–1140
24. Lupas, A. (1996) *Trends Biochem. Sci.* 21, 375–382
25. Mezey, A. W., Dakeji, S. I., Olsen, O., Bredt, D. S., Lim, W. A., and Prehoda, K. E. (2001) *Mol. Cell* 8, 1291–1301
26. Tavares, G. A., Panepeci, E. P., and Brunger, A. T. (2001) *Mol. Cell* 8, 1315–1325
27. Nusrat, S., and Weis, W. (2000) *Mol. Cell* 273, 95, 715–719
28. Marfatia, S. M., Leu, R. A., Branton, D., and Chishti, A. H. (1995) *J. Biol. Chem.* 270, 6406–6412
29. Fanning, A. S., Jameson, B. J., Jesaitis, I. A., and Anderson, J. M. (1998) *J. Biol. Chem.* 273, 29745–29753
30. Imamura, Y., Itoh, M., Maeno, Y., Tsukita, S., and Nagafuchi, A. (1999) *J. Cell Biol.* 144, 1311–1322
31. Kaulaulya, P., Reichert, M., and Hunziker, W. (2001) *FEBS Lett.* 505, 92–96
The Tight Junction Protein Occludin and the Adherens Junction Protein \( \alpha \)-Catenin Share a Common Interaction Mechanism with ZO-1
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*J. Biol. Chem.* 2005, 280:3747-3756.
doi: 10.1074/jbc.M411365200 originally published online November 16, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M411365200

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