The Coiled-coil Domain of Occludin Can Act to Organize Structural and Functional Elements of the Epithelial Tight Junction*

Received for publication, March 21, 2000, and in revised form, June 20, 2000 Published, JBC Papers in Press, July 7, 2000, DOI 10.1074/jbc.M002450200

Asma Nusrat‡, Jason A. Chen‡, Chris S. Foley‡, Tony W. Liang§, Jeffrey Tom¶, M. Cromwell†, Cliff Quan‡, and Randall J. Mrsny**

From the ‡Epithelial Pathobiology Research Unit, Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia 30322 and the Departments of §Mimsunology, ¶Bioorganic Chemistry, and ¶Pharmaceutical Research and Development, Genentech, Inc., South San Francisco, California 94080

Occludin is an integral membrane protein that has been suggested to play a role in the organization and dynamic function of the epithelial tight junction (TJ). A number of other proteins have also been described to localize to the TJ. We have used a novel bait peptide method to investigate potential protein-protein interactions of the putative coiled-coil domain of occludin with some of these other TJ proteins. A 27-amino acid peptide of the human occludin sequence was synthesized, biotinylated at the N terminus, and modified to contain a photoactive moiety at either its hydrophobic or hydrophilic surface. These bait peptides were α-helical in solution, characteristic of coiled-coil structures. Photoactivation studies in the presence and absence of control peptides were used to assess the potential interactions in polarized sheets of a human intestinal cell line T84. Although a large number of proteins associated with the TJ or that are known to be involved in regulatory events of epithelial cells failed to be specifically labeled, occludin itself, ZO-1, protein kinase C-ζ, c-Yes, the regulatory subunit of phosphatidylinositol 3-kinase, and the gap junction component connexin 26 were specifically labeled. Our data demonstrate the potential of one specific domain of occludin, contained within 27 amino acids, to coordinate the binding of proteins that have been previously suggested to modulate TJ structure and function.

Over the last decade a number of proteins have been identified that localize to the tight junction (TJ)1 structures of epithelial cells. Possible functional interactions between these proteins have been described (reviewed in Ref. 1). Critical extracellular interactions in TJs have been attributed to two transmembrane proteins, claudin(s) and occludin (2). Although it has been suggested that claudins recruit occludin to TJ sites (3), several recent studies have suggested instead that occludin dynamically regulates claudin-based TJ strands. Transfection of occludin mutants lacking either the intracellular (4) or extracellular (5) domains induces disruption of epithelial barrier properties. In addition, TJ barrier function is also influenced by incubation with peptides containing the two extracellular loop amino acid sequences (6–8). As yet, analogous studies have not been described for claudin(s). Finally, disruption of TJ function mediated by the constitutive activation of Raf-1 is associated with down-regulation of occludin and claudin-1 expression, an effect that can be reversed by the reintroduction of occludin expression that in turn restores claudin-1 protein levels (9).

Human occludin is approximately 65 kDa with what appears to be a 65-amino acid cytosolic N terminus, two extracellular loops of 46 and 48 amino acids separated by a 10-amino acid cytosolic loop, and a C-terminal tail of approximately 255 amino acids (10). Both the N- and C-terminal domains have a large number of serine and threonine residues, and the functionally active form of the protein localizing to the TJ appears to be hyperphosphorylated at serine and threonine residues (11, 12). Recent studies have also suggested a potential role for tyrosine phosphorylation in the dynamic regulation of occludin (13). The long intracellular C-terminal tail of occludin has been proposed to have interactions with TJ-associated proteins such as the zonula occludens proteins ZO-1, ZO-2, and ZO-3 (14, 15). These proteins, in turn, all contain conserved domains consisting of guanylate kinase, Src homology SH3 structures, PSD-95,DlgA, and ZO-1-like binding sites, and proline-rich sequences. The SH3, PSD-95, DlgA, and ZO-1-like, and proline-rich domains appear to be capable of interacting with other intracellular proteins, such as occludin, that also localize to epithelial TJs. Within the putative 150-amino acid ZO-1 binding domain of occludin is a 27-amino acid stretch with hydrophobic residues clustered in a pattern consistent with a coiled-coil structure (10).

We have used a novel bait peptide method to determine the potential interaction of proteins known to localize to the TJ, associate with occludin, or regulate TJ function. Our results demonstrate for the first time that occludin can specifically interact with itself through its coiled-coil domain and suggest that the established interaction between ZO-1 and occludin may occur through this same domain. Additionally, the coiled-coil domain of occludin appears to act as a site for specific interactions of several potential regulatory proteins, suggesting a pivotal role for occludin in the coordinated associations of TJ components in epithelial cells. Further, a potential link between a specific domain of occludin and a gap junction element (connexin 26) may have been identified.

EXPERIMENTAL PROCEDURES

Peptide Bait Synthesis—Peptides (see Table I) were synthesized on an automated Pioneer Peptide Synthesizer (PE/ABI) with Fmoc-pro-
Characteristics of coiled-coil bait peptides

| Peptide sequence | Ocludin domain | Code name |
|------------------|----------------|-----------|
| Biotin-LQEYKSLQSELDF*NKELSRLDKELDDYRE-NH2 | Hydrophilic surface of coiled-coiled domain Leu440-Glu469 | CCD-In |
| Biotin-LQEYKSLQSELDFN*KELSRLDKELDDYRE-NH2 | Hydrophilic surface of coiled-coiled domain Leu440-Glu469 | CCD-Out |

*Table 1: Characteristics of coiled-coil bait peptides*

Potential Protein Interactions with Ocludin

Standard one-letter amino acid codes are used except where F* indicates benzoyl phenylalanine (Advanced ChemTech) amino acid was covalently attached to HBTU-HOBT (Sigma) is incorporated into peptides at the N terminus using HBTU-HOBt/DIPEA in MeSO4. Peptide residues were cleaved with a 1-h exposure of a 95% trifluoroacetic acid/2.5% trisopropylsilane/2.5% H2O mixture. Released peptides were purified by preparative reversed-phase C18 high performance liquid chromatography, characterized by electrospray ionization mass spectroscopy (Sciex API100), and lyophilized to dryness.

Biophysical Characterization of Peptide Bait—CD spectra were collected on an Aviv 60DS spectropolarimeter. Near UV CD spectra (400–250 nm) were obtained in 0.2-nm increments with a 0.5-nm bandwidth and a 1-s time constant for samples in a 1-cm pathlength cell and far UV spectra (250–190 nm) were collected in 0.2-nm increments with a 0.5-nm bandwidth and a 1-s time constant in a 0.05-cm pathlength cell. All spectra were digitally smoothed using the Savitsky-Golay algorithm (17), corrected for concentration, and normalized to units of mean residue weight ellipticity ($\Theta_{MRW}$) using the following relationship.

$$\Theta_{MRW} = \frac{\Theta_{obs}(\text{MW}_{monomer}/\text{MW}_{monomer})}{10^3/\text{deg}} \quad \text{(Eq. 1)}$$

where $\Theta_{obs}$ is the observed ellipticity, $\text{MW}_{monomer}$ is the molecular weight of the monomer, $n_{monomer}$ is the number of amino acids in the monomer, $d$ is the pathlength of the cell (in cm), and $c$ is the concentration of the sample in the cell (in mg/ml).

Fishing with Peptide Bait—T84 cells (American Type Culture Collection) were grown on 45 cm2 collagen-coated permeable supports, and transepithelial resistance values were measured with a voltmeter as described previously (18). Briefly, the apical and basolateral reservoirs of filters were connected to calomel and Ag-AgCl electrodes via agar bridge. Transepithelial resistance values were measured with a voltmeter as described previously (18). Briefly, the apical and basolateral reservoirs of filters were connected to calomel and Ag-AgCl electrodes via agar bridge. Transepithelial resistance values were measured with a voltmeter as described previously (18). Briefly, the apical and basolateral reservoirs of filters were connected to calomel and Ag-AgCl electrodes via agar bridge.

RESULTS

CCD-In and CCD-Out Peptides Have $\alpha$-Helical Structures in Solution—The proposed coiled-coil domain of human ocludin extends approximately from Leu440 to Gln469 (10). It resides within the 150 C-terminal amino acids previously shown to be involved in the association of ocludin with ZO-1 and its localization to the TJ (21). Bait peptides synthesized as a 27-amino acid sequence from this region (Table I) were found to have solution characteristics by circular dichroism consistent with a significant $\alpha$-helical content (Fig. 1). The presence of biotin or a photoactive residue did not disrupt the $\alpha$-helical nature of these peptides in solution (data not shown). By comparison, a 26-amino acid synthetic linear peptide of the V3 loop of HIV-1 MN gp120 failed to show such a strong helical content.

A Small Population of Proteins Is Specifically Labeled by Ocludin Coiled-coil Bait Peptides—The bait peptide fishing
sequence from Leu440 to Glu469, N-terminal biotinylated and C-terminal bait peptides were comprised of 27 amino acids of the human occludin units of mean residue weight ellipticity. Both CCD-In and CCD-Out smoothed, corrected for concentration differences, and normalized to three scans following background spectrum subtraction) were digitally A structures in solution.

a the SEL-CON fitting program. Calculated changes in secondary structure resulting from polymerization events reactive moiety previously inactivated to eliminate the possibility of CD studies were performed with bait peptides that had the photo-reactive moiety previously inactivated to eliminate the possibility of amidated: Biotin-LQEYKSLQSELDEFNKELSRLDKELDDYRE-NH₂.

CD studies were performed with bait peptides that had the photo-reactive moiety previously inactivated to eliminate the possibility of near-UV and far-UV CD spectra (mean of three scans following background spectrum subtraction) were digitally smoothed, corrected for concentration differences, and normalized to units of mean residue weight ellipticity. Both CCD-In and CCD-Out bait peptides were comprised of 27 amino acids of the human occludin sequence from Leu⁴⁴⁰ to Glu⁶⁶⁹. N-terminal biotinylated and C-terminal amidated: Biotin-LQEYKSLQSELDEFNKELSRLDKELDDYRE-NH₂.

CD studies were performed with bait peptides that had the photo-reactive moiety previously inactivated to eliminate the possibility of changes in secondary structure resulting from polymerization events during analysis. B, secondary structure calculations performed using the SEL-CON fitting program. Calculated α-helical content (asterisk) agrees with values determined from changes in observed ellipticity at 222 nm.

FIG. 1. CCD-In and CCD-Out bait peptides have α-helical structures in solution. A, near-UV and far-UV CD spectra (mean of three scans following background spectrum subtraction) were digitally smoothed, corrected for concentration differences, and normalized to units of mean residue weight ellipticity. Both CCD-In and CCD-Out bait peptides were comprised of 27 amino acids of the human occludin sequence from Leu⁴⁴⁰ to Glu⁶⁶⁹. N-terminal biotinylated and C-terminal amidated: Biotin-LQEYKSLQSELDEFNKELSRLDKELDDYRE-NH₂.

approach used in our studies showed striking selectivity of labeling of protein targets. Confluent, high resistance monolayers of T84 cells were slightly permeabilized to allow the penetration of bait peptides and control peptides. After photoactivation and enrichment for Triton-insoluble membranes, samples were separated by SDS-PAGE, blotted, and probed for biotinylation using streptavidin-peroxidase (Fig. 2). These blots show that only a limited number of biotinylated bands were specifically labeled as assessed by potent competition with 10-fold excess control peptide. Additionally, there were some similarities but also striking differences between the target labeling patterns (Fig. 2) observed for CCD-In (hydrophobic surface photoactive bait) and CCD-Out (hydrophilic surface photoactive bait) peptides. Further enrichment of biotin-positive conjugates was performed using monomeric avidin beads. Initial immunoblot studies were performed to identify known proteins previously shown to localize to the TJ or have regulatory actions on TJ function. In each case, whole cell lysates were probed with the antibody used to verify the presence of the protein in question in our T84 cell monolayers.

Proteins present in our T84 lysates that were not positive for biotin labeling included Claudin-1, ZO-2, ZO-3, human junctional adhesion molecule, connexin 32, actin, Lyn, c-Src, rho, phospholipase Cγ, protein serine/threonine phosphatase 1, protein-tyrosine phosphatase β1, and caveolin-1 (data not shown). A discrete population of regulatory and TJ proteins, however, were positively identified in this immunoblot screen. Those positive identifications are noted in Fig. 2 and characterized further in studies described below. Several unidentified proteins labeled by this method are shown. The identities of these proteins are currently under investigation. Some of these proteins may represent associations involving the synthesis, trafficking, or elimination of occludin and not directly involved in structure/function relationships of the epithelial TJ.

Occludin-Occludin Interactions Occur through the Coiled-coil Domain—Bait peptides having the photoactive residue at the hydrophobic surface (CCD-In) or at the hydrophilic surface (CCD-Out) both bound specifically to occludin (Fig. 3A). Binding to both of these peptides was competed in the presence of excess control peptide. Two prominent specifically labeled biotin-positive bands were positively identified as occludin by Western immunostaining (Fig. 3A, arrowheads (arrows 1, 2, 3, 4, 5, 6)), PKC-ζ (arrow 4), c-Yes (arrow 5), and connexin 26 (arrow 6).
precipitation experiments verified that both the 65- and 80-kDa bands contained the first 9 amino acids of the N-terminal sequence of human occludin (data not shown). Although variations in antibody binding and biotin accessibility might occur, these results suggest that the bait peptides used interact more efficiently with the hyperphosphorylated forms of occludin compared with occludin in its basal state lacking significant phosphorylation. Neither bait peptide specifically labeled claudin-1 (data not shown), another integral membrane protein of the TJ complex (22).

Bait peptide-occludin complexes isolated by immunoprecipitation with an anti-occludin antibody were cleaved using chymotrypsin (Fig. 3B). Biotinylated peptide fragments were identified following SDS-PAGE with avidin-horseradish peroxidase having approximately molecular masses of 75 kDa (Fig. 3B, arrow 1), 27 kDa (arrow 2), 12 kDa (arrow 3), and 6 kDa (arrow 4). The most intense biotin-positive fragment had a molecular mass of approximately 12 kDa (Fig. 3B). This observed mass is consistent with the combined masses of the bait peptide (~4 kDa) plus a peptide fragment of occludin containing the coiled-coil domain that could be released by chymotrypsin cleavage at residues Phe⁶³⁶ and Tyr⁶⁷⁴. This is not a unique solution because other combinations of chymotrypsin cleavage of the bait peptide and occludin could be imagined to yield a similar apparent molecular mass. Exact identification of composition, however, could not be verified by amino acid sequencing because of technical limitations of the procedure required to isolate sufficient material for analysis.

**ZO-1 Interacts with Both Coiled-coil Domain Bait Peptides**—ZO-1 selectively interacted with both CCD-In and CCD-Out bait peptides (Fig. 4). Under the labeling conditions used, the CCD-In bait peptide labeled a 220-kDa ZO-1 band more intensely than did the CCD-Out peptide. ZO-2 and ZO-3 are other members of the MAGUK family of proteins enriched at TJ complexes along with ZO-1 (14, 15), and these proteins appear to be involved in the Rho-regulated actin coupling of actin cables to the TJ (23). ZO-2, ZO-3, actin, and Rho, however, were not observed to interact specifically with either the CCD-In or the CCD-Out bait peptides (data not shown).

**Several Regulatory Proteins Interact with the Coiled-coil Domain Bait Peptides**—An 85-kDa component of PI 3-kinase was identified by Western blotting as a protein that associated with the T84 cell membranes isolated by ultracentrifugation following reaction with photo-activate bait peptides (CCD-In and CCD-Out) were incubated with monomeric avidin beads to isolate biotinylated peptide-protein complexes (lane H of panels I and II). Specificity of association between bait peptides and occludin was verified by preincubation with a ten-fold excess of control peptide (lane C of panels I and II). Occludin Western blot of T84 cell lysates is shown in panel III. B, occludin associated with bait peptide was isolated by immunoprecipitation with antibodies to occludin, cleaved by chymotrypsin incubation, separated by SDS-PAGE, transferred onto nitrocellulose, probed with horseradish peroxidase-conjugated streptavidin, and visualized by enhanced chemiluminescence. The avidin banding pattern of occludin in the absence (panel I) and presence (panel II) of chymotrypsin is shown.

**FIG. 3. Occludin binds to itself through coiled-coil domain.** A, T84 cell membranes isolated by ultracentrifugation following reaction with photo-activate bait peptides (CCD-In and CCD-Out) were incubated with monomeric avidin beads to isolate biotinylated peptide-protein complexes (lane H of panels I and II). Specificity of association between bait peptides and occludin was verified by preincubation with a ten-fold excess of control peptide (lane C of panels I and II). Occludin Western blot of T84 cell lysates is shown in panel III. B, occludin associated with bait peptide was isolated by immunoprecipitation with antibodies to occludin, cleaved by chymotrypsin incubation, separated by SDS-PAGE, transferred onto nitrocellulose, probed with horseradish peroxidase-conjugated streptavidin, and visualized by enhanced chemiluminescence. The avidin banding pattern of occludin in the absence (panel I) and presence (panel II) of chymotrypsin is shown.

**FIG. 4. Association of ZO-1 with occludin bait peptides.** A, confluent monolayers of T84 cells were disrupted by nitrogen cavitation and incubated with CCD-In (panel I) or CCD-Out (panel II) bait peptide in the absence (H) or presence (C) of a ten-fold excess of a control peptide. Membranes were pelleted and biotin-positive proteins were selected by precipitation onto avidin beads and separated by SDS-PAGE prior to immunoblot analysis. Bound primary antibodies were visualized by substrate reactions for horseradish peroxidase covalently associated with a secondary antibody. Binding of ZO-1 to both the CCD-In and CCD-Out peptides was competed in the presence of 10-fold excess control peptides. A Western blot for ZO-1 present in a whole T84 cell lysate is shown in panel III.

**FIG. 5. Association of signaling proteins with the CCD bait peptides.** Confluent monolayers of T84 cells were disrupted by nitrogen cavitation and incubated with CCD-In (panel I) or CCD-Out (panel II) bait peptide in the absence (H) or presence (C) of a ten-fold excess of a control peptide. Biotin-positive proteins present in pelleted membrane preparations were enriched by precipitation onto avidin beads and separated by SDS-PAGE prior to immunoblot analysis. Bound primary antibodies were visualized by substrate reactions for horseradish peroxidase covalently associated with a secondary antibody. Western blots for total expression in whole cell lysates for the respective proteins are shown in panel III. The 85-kDa component of PI 3-kinase binds to peptides in both the CCD-In and CCD-Out configuration and is competed off in the presence of excess control peptides. However, specific binding of PKC-ζ and c-Yes was observed only with the CCD-In bait peptide. No specific binding was observed for other candidate signal transduction proteins (e.g. Rho, c-Src, and protein serine/threonine phosphatase 1).

Both the CCD-In and CCD-Out bait peptides (Fig. 5). This protein contains two SH2 domains and an SH3 domain. It associates with and serves as a substrate for activated growth factor receptor kinases and has been suggested to play a regulatory role by serving as a link between PI 3-kinase and ligand-activated receptors (24). Phospholipase C, PKC, the GTP-binding proteins Rac and Rho, as well as the serine-
threonine kinase Akt/protein kinase B have been identified as downstream effectors of PI 3-kinase activity (reviewed in Ref. 25). Although all of these effector molecules were detectable in T84 cell lysates, only PKC-ζ was also observed to bind specifically with either of the bait peptides. Additionally, this labeling was specific for the CCD-In bait peptide, suggesting that PKC-ζ and the coiled-coil domain of occludin interact at its hydrophobic surface. This putative interaction may account for the observed localization of PKC-ζ to the epithelial TJ (reviewed in Ref. 25).

The combined actions of PI 3-kinase and PKC-ζ have been suggested to regulate the actin cytoskeleton of cells (reviewed in Ref. 24). Activation of PI 3-kinase is sufficient to disrupt epithelial polarization (26). In addition, activation of the tyrosine kinase pathway has also been proposed to modulate TJ function (reviewed in Ref. 25). One of these kinases, c-Yes, was found to specifically interact with only the CCD-In bait peptide (Fig. 5), suggesting an interaction through the hydrophobic surface of the coiled-coil domain of occludin. Although Western blotting studies (data not shown) verified the expression of other kinases (c-Src and lyn) in T84 whole cell lysates, these nonreceptor tyrosine kinases did not interact with the bait peptides.

**Connexin 26 Interacts with the Hydrophilic Surface of the Bait Peptide**—Occludin-induced TJ strands have been observed to occasionally associate with gap junction structures (27). Three proteins, connexin 26, connexin 32, and connexin 43, have been identified as prominent structural components of the gap junction. Western blot examination of T84 lysates probed with our bait peptides resulted in the singular identification of specific connexin 26 labeling with the CCD-Out or hydrophilic surface probe (Fig. 6, panels I and II). This specific labeling was observed in the membrane fractions obtained from confluent T84 monolayers. Despite its expression as verified by Western blotting of whole cell lysates, no labeling for connexin 26 was observed using these bait peptides (Fig. 6, panels I and III). The significance of a potential association between the hydrophilic surface of the coiled-coil domain of occludin and connexin 26 is as yet unclear. ZO-1 has previously been proposed to interact with connexin 43 (28). However, we could not identify connexin 43 in Western blots of T84 cell lysates (data not shown). Although T84 cells have been shown to contain functional gap junctions (29), it is unclear which of the connexin proteins are expressed in a stable fashion by these cells under confluent growth conditions.

**DISCUSSION**

Coiled-coil domains have been identified as a potential site for protein-protein contacts, and the interactions between proteins at these contact sites can be emulated using peptides (30). Our approach using biotinylation to track and isolate chemical conjugates of coiled-coil bait peptides following activation of a photoactive group is similar to previous studies that have employed synthetic peptides to mimic and characterize specific protein-protein or protein-peptide interactions. Synthetic peptides corresponding to C-terminal domains of Gα have been used in a permeabilized cell system to study receptor-G protein-effector coupling (31). A photolabile, biotin-conjugated form of α-melanocyte-stimulating hormone has been used to identify and partially purify ligand-receptor complexes (32). A biotinylated form of the neuropeptide somatostatin has been used for receptor purification and localization following photoaffinity cross-linking (33). T cell receptor interactions have been studied using a biotinylated, photoactive peptide (34).

Like these previous studies, our current results have demonstrated that this approach can be used to identify potential protein-peptide interactions. We have found that this bait peptide fishing approach can be used in place of technically challenging immunoprecipitation methods to identify potential binding partners of an integral membrane protein.

We have investigated potential contacts of the coiled-coil domain of an integral membrane protein that has been identified as a component of the TJ complex. Several similar synthetic peptides have previously been used to model the coiled-coil structures present in a number of biological systems (35). In our studies, a 27-amino acid-long synthetic peptide that emulates the coiled-coil region of human occludin was synthesized and chemically modified to contain an N-terminal biotin. An amino acid residue near the middle of either the hydrophilic or hydrophobic surface of the coiled-coil structure was replaced with a residue that could be photo-activated to form a covalent attachment site. Bait peptides were incubated with membranes isolated from polarized, high resistance T84 cell monolayers. After photoactivation samples were separated by SDS-PAGE, blotted, and probed for biotinylated structures.

These blots showed that only a small number of biotinylated bands were specifically labeled. Western blotting demonstrated that primary biotin-positive bands were recognized by antibodies to occludin, ZO-1, c-Yes, the regulatory (p85) subunit of PI 3-kinase, PKC-ζ, and connexin 26. However, a number of proteins present in our T84 lysates that have been demonstrated to localize at or modulate the function of TJs failed to be selectively labeled by the coiled-coil bait peptides. Proteins not positive for biotin labeling using these bait peptides included claudin-1, ZO-2, ZO-3, human junctional adhesion molecule, connexin 32, actin, lyn, c-Src, Rho, phospholipase Cγ, protein serine/threonine phosphatase 1, protein-tyrosine phosphatase β1, and caveolin-1 (data not shown). Additionally, protein labeling was selective for CCD-In (hydrophilic surface photoactive bait) and CCD-Out (hydrophilic surface photoactive bait) peptides. For example, occludin, ZO-1 and PI 3-kinase p85 were labeled by both CCD-In and CCD-Out peptides. PKC-ζ and c-Yes were labeled only by the CCD-In peptide, and connexin 26 was labeled only by the CCD-Out peptide. Thus, the labeling approach that we have described appears to be selective by several criteria for proteins that might potentially interact with the coiled-coil domain of occludin.

Our results have provided information that should increase...
Potential Protein Interactions with Occludin

Our understanding of how occludin can function as a TJ component. Previous studies evaluating the impact of C-terminal truncation on occludin function, which also removed the coiled-coil domain, suggested that this portion of the protein may be involved in oligomeric assemblies (36). For the first time the coiled-coil domain of this protein has now been demonstrated to have selective association with itself and several proteins that may play a structural and/or functional role in the TJ. Zo-1 interactions with occludin have previously been shown to occur within a region of occludin from Asn373 to Thr522 (21). Our data have now identified one potential site for this interaction to involve the coiled-coil domain (Leu440–Glu469) nestled within this large region. Other members of the MAGUK family of proteins, ZO-2 (37) and ZO-3 (38), did not appear to specifically interact with the coiled-coil domain bait peptides used in these studies. Together, these interactions between occludin with itself and with Zo-1 improve our understanding of how these proteins can establish the supramolecular structures that have been suggested for the TJ (discussed in Ref. 14). Our findings suggest, for the first time, that the coiled-coil domain of occludin is a coordinating site for these oligomeric structures and that occludin and Zo-1, but not Zo-2 or ZO-3, participate in these contacts. Zo-1 has been suggested to interact with occludin, Zo-2, and F-actin (39), and both Zo-2 and Zo-3 interact with Zo-1/occludin complexes (38, 40). Our data now stipulate that these interactions can occur through an organizing structure on the occludin protein, its 27-amino acid coiled-coil domain, where protein-protein contacts occur through both the hydrophobic and hydrophilic surfaces of this domain.

We also demonstrated that several proteins associated with kinase function can also selectively interact with the coiled-coil domain of occludin. This is important information because phosphorylation events control the development and dynamics of functional TJ complexes (41). Occludin localized to TJs is hyperphosphorylated at serine/threonine residues (11, 12). Zo-1 also appears to be phosphorylated at serine/threonine residues (42). Disruption of epithelial barrier properties has been correlated with either a decrease in serine/threonine phosphorylation or an increase in tyrosine phosphorylation of these TJ components (43–45). We have now demonstrated for the first time the specific interaction of three proteins involved in protein phosphorylation, the nonreceptor tyrosine kinase c-Yes, PKC-ζ, and the p85 regulatory subunit of PI 3-kinase, with a singular domain of occludin. The 85-kDa regulatory subunit of PI 3-kinase associates with and serves as a substrate for activated growth factor receptor tyrosine kinases (24). PI 3-kinase appears to be involved in the polymerization of actin for activated growth factor receptor tyrosine kinases (24). PI 3-kinase and PKC-ζ in protein phosphorylation, the nonreceptor tyrosine kinase may appear to control the development and dynamics of functional TJ complexes (46). Together, these interactions between occludin with itself and with Zo-1 improve our understanding of how these proteins can establish the supramolecular structures that have been suggested for the TJ (discussed in Ref. 14). Our findings suggest, for the first time, that the coiled-coil domain of occludin is a coordinating site for these oligomeric structures and that occludin and Zo-1, but not Zo-2 or ZO-3, participate in these contacts. Zo-1 has been suggested to interact with occludin, Zo-2, and F-actin (39), and both Zo-2 and Zo-3 interact with Zo-1/occludin complexes (38, 40). Our data now stipulate that these interactions can occur through an organizing structure on the occludin protein, its 27-amino acid coiled-coil domain, where protein-protein contacts occur through both the hydrophobic and hydrophilic surfaces of this domain.

Our findings concerning the specific associations of several kinases to the bait peptides used in this study provide the first data to suggest a highly selective interaction between occludin and regulatory proteins capable of modulating the function of the TJ. Further, our studies identify a singular domain of occludin to be involved in these interactions and identifies a potential dynamic relationship between these specific kinase-related proteins and TJ function. A further point can be made about how these interactions can occur. Although the regulatory subunit of PI 3-kinase can interact with both interfacial surfaces of the coiled-coil domain of occludin, PKC-ζ and c-Yes interact only with the hydrophobic surface.

Finally, connexin 26 was identified to interact in a specific manner at the hydrophilic interface of the intracellular coiled-coil domain of occludin. Connexin 26 is a component of gap junction structures. The possibility of an occludin-connexin 26 interaction provides an interesting explanation for several previously reported observations that suggest a proximity of TJ and gap junction structures (3, 5). A recent study has shown connexin 32 to directly interact with occludin (52). Our data localized the specific interaction of connexin 26 to a 27-amino acid domain of occludin. At present, it is unclear how connexin 32 might interact with occludin because we could not demonstrate an interaction between this protein and the coiled-coil occludin bait peptides, despite its expression in T84 cell monolayers.

Of the proteins identified in this study to specifically interact with our bait peptides, only occludin, the p85 regulatory component of PI 3-kinase, and connexin 26 appear to have potential coiled-coil domains (all protein sequences were analyzed using Lupas’ algorithm of the PSORT program). Because the current paradigm of coiled-coil contact interaction describes the association of similar α-helical domains, it is unclear how these proteins that lack this structural attribute might interact with the coiled-coil domain of occludin. In vitro studies with the bait peptides have shown them to form oligomers under the solution conditions used in our studies. The association states of occludin and interactions with other proteins may also depend upon the phosphorylation state of occludin or these other proteins involved because several kinases have now been identified to directly interact with occludin at this site. It is also important to note that our studies identifying proteins with potential interactions with the coiled-coil domain of occludin do not imply that these same proteins cannot have other specific interaction sites on the occludin molecule.

In summary, the data obtained to date using our bait peptide fishing approach support the concept that a component of the TJ, occludin, may act to coordinate elements of the actin cytoskeletal and signaling pathways to this structure in polarized epithelia through its coiled-coil domain. Such a function may be similar to that previously observed for integrins (53) and caveolin (54), which can act as membrane-anchored scaffolding proteins. Occludin may also be acting similarly in concert with another TJ protein known as cingulin. This protein has recently been shown to contain putative coiled-coil domains, self-associate, and interact specifically with Zo-1, Zo-2, and Zo-3 (55).

Acknowledgment—We thank Dr. Danxi Li for reading this manuscript.

REFERENCES

1. Yap, A. S., Mullin, J. M., and Stevenson, B. R. (1998) J. Membr. Biol. 163, 159–167.
2. Tsukita, S., and Furuse, M. (1999) Trends Cell Biol. 9, 268–273.
3. Furuse, M., Sasaki, H., Fujimoto, K., and Tsukita, S. (1996) J. Cell Biol. 143, 391–401.
4. Baldo, M. S., Whitney, J. A., Flores, C., Gonzalez, S., Cerijevi, M., and Matter, K. (1996) J. Cell Biol. 134, 1031–1049.
5. Bamforth, S. D., Kruel, U., Wolberg, H., Engelhardt, B., and Risau, W. (1999) J. Cell Biol. 151, 1879–1888.
6. Wong, V., and Gumbiner, B. M. (1997) J. Cell Biol. 136, 39–409.
7. Van Itallie, C. M., and Anderson, J. M. (1997) J. Cell Sci. 116, 1113–1121.
8. Laca-Vieira, F., Jaeger, M. M., Farshori, P., and Kachar, B. (1999) J. Membr. Biol. 168, 289–297.
9. Li, D., and Mrsnj, R. J. (2000) J. Cell Biol. 148, 791–800.
10. Ando-Akatsuka, Y., Saitou, M., Hirase, T., Kishi, M., Sakakibara, A., Itoh, M., Yonemura, S., Furuse, M., and Tsukita, S. (1996) J. Cell Biol. 133, 43–47.
11. Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y., and Tsukita, S. (1997) J. Cell Biol. 137, 1393–1401.
12. Wong, V. (1997) Am. J. Physiol. 42, C1859–C1867.
13. Chen, Y.-h., Lu, Q., Schneeberger, E. E., and Gordon, D. A. (2000) Mol. Biol. Cell 11, 849–862.

R. J. Mrsnj, J. Chen, T. W. Liang, J. Tom, C. Quan, and A. Nusrat, manuscript in preparation.
Potential Protein Interactions with Occludin

14. Goodenough, D. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 319–321
15. Wittchen, E. S., Haskins, J., and Stevenson, B. R. (1999) J. Biol. Chem. 274, 35179–35185
16. Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149–2154
17. Gorry, P. A. (1999) Anal. Chem. 72, 570–573
18. Merrifield, R. B. (1963) J. Am. Chem. Soc. 85, 2149–2154
19. Towbin, H. T. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4150
20. Kaoutzani, P., Parkos, C. A., Delp-Archer, C., and Madara, J. L. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 10629–10633
21. Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S., and Tsukita, S. (1994) J. Cell Biol. 127, 1617–1626
22. Furuse, M., Fujita, K., Hiragi, T., Fujimoto, K., and Tsukita, S. (1996) J. Cell Biol. 134, 1539–1550
23. Nusrat, A., Giry, M., Turner, J. R., Colgan, S. P., Parkos, C. A., Carnes, D., Lemchek, E., Boquet, P., and Madara, J. L. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 10629–10633
24. Denker, B. M., and Nigam, S. K. (1998) Am. J. Physiol. 274, F1–F9
25. Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J., and Parise, L. V. (1997) J. Cell Biol. 137, 1127–1136
26. Giepmans, B. N. G., and Moolenaar, W. H. (1998) Curr. Biol. 8, 931–934
27. Chanson, M., White, M. M., and Garber, S. S. (1996) Am. J. Physiol. 271, C531–C539
28. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997) Nature 387, 426–430
29. Rasenick, M. M., Lazarievic, M., Watanabe, M., and Hamm, H. E. (1993) Methods 5, 352–357
30. Ahmed, A. R., Olivier, G. W., Adams, G., Erskine, M. E., Kinsman, R. G., Branch, S. K., Moss, S. H., Notarianni, L. J., and Pouton, C. W. (1992) Methods 5, 377–382
31. Rasenick, M. M., Lazarevic, M., Watanabe, M., and Hamm, H. E. (1993) Methods 5, 352–357
32. Ahmed, A. R., Olivier, G. W., Adams, G., Erskine, M. E., Kinsman, R. G., Branch, S. K., Moss, S. H., Notarianni, L. J., and Pouton, C. W. (1992) J. Cell Biol. 103, 6072–6076
33. Schonbrunn, A., Lee, A. B., and Brown, P. J. (1993) Endocrinology 132, 146–154
34. Romero, P., Casanova, J. L., Cerottini, J. C., Maryanski, J. L., and Luescher, I. F. (1999) J. Exp. Med. 177, 1247–1256
35. O'Shea, E. K., Rutkowski, R., and Kim, P. S. (1989) Science 243, 538–542
36. Chen, Y.-h., Merzendorf, C., Paul, D. L., and Goodenough, D. A. (1997) J. Biol. Chem. 335, 10629–10633
37. Jesaitis, L. A., and Goodenough, D. A. (1994) J. Cell Biol. 124, 949–961
38. Haskins, J., Gu, L., Wittchen, E. S., Hibbard, J., and Stevenson, B. R. (1998) J. Cell Biol. 141, 199–208
39. Fanning, A. S., Jameson, B. J., Jesaitis, L. A., and Anderson, J. M. (1998) J. Biol. Chem. 273, 29745–29753
40. Takeda, H., Nagafuchi, A., Yonemura, S., Tsukita, S., Behrens, J., Birchmeier, W., and Tsukita, S. (1995) J. Cell Biol. 131, 1839–1847
41. Stevenson, B. R., Siliciano, M. S., Mosek, M. S., and Goodenough, D. A. (1986) J. Cell Biol. 103, 755–766
42. Singer, R. L., Stevenson, B. R., Woo, P. L., and Firestone, G. L. (1994) J. Biol. Chem. 269, 16108–16115
43. Staddon, J. M., Herrenknecht, K., Smales, C., and Rubin, L. L. (1995) J. Cell Sci. 108, 609–619
44. Takeda, H., and Tsukita, S. (1995) Cell. Struct. Funct. 20, 307–319
45. Takeda, H., and Tsukita, S. (1995) Eur. J. Cell Biol. 76, 185–191
46. Massoumi, R., and Sjolander, A. (1998) Eur. J. Cell Biol. 76, 185–191
47. Ptaszynski, A., Beattie, G. M., Mally, M. I., Cirulli, V., Lopez, A., and Hayek, A. (1997) J. Cell Biol. 137, 1127–1136
48. Stuart, R. O., and Nigam, S. M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6072–6076
49. Dodane, V., and Kachar, B. (1996) J. Membr. Biol. 149, 199–209
50. Sniegowski, P., and Nigam, S. M. (1995) J. Membr. Biol. 149, 199–209
51. Gomez, J., Garcia, A., Borlado, L., Bonay, P., Martinez, C., Silva, A., Fresno, M., Carrera, A. C., Eicher-Streiber, C., and Rebollo, A. (1996) J. Membr. Biol. 149, 199–209
52. Kojima, T., Sawada, N., Chiba, H., Kokai, Y., Yamamoto, M., Urban, M., Lee, G. H., Hertzberg, E. L., Mohchzuki, Y., and Spray, D. C. (1999) Biochem. Biophys. Res. Commun. 266, 222–229
53. Giancotti, F. G., and Ruoslahti, E. (1999) Science 285, 1028–1032
54. Li, S., Couet, J., and Lisanti, M. P. (1996) J. Biol. Chem. 271, 29182–29190
55. Cordenonsi, M., D’Atri, F., Hammer, E., Parry, D. A. D., Kendrick-Jones, J., Shore, D., and Citi, S. (1999) J. Cell Biol. 147, 1569–1581