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Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1

(cell junction/zonula occludens/Madin–Darby canine kidney epithelial cell)

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ABSTRACT **ZO-1 is a 210- to 220-kDa peripheral membrane protein associated with the cytoplasmic surface of the epithelial tight junction. Because ZO-1 may interact with other unidentified tight junction proteins, we have looked for other polypeptides that bind to ZO-1. A 160-kDa polypeptide was identified that coimmunoprecipitates with ZO-1 from detergent extracts of metabolically labeled Madin–Darby canine kidney (MDCK) cells. This polypeptide appears to be distinct from ZO-1, rather than a degradation product, by several criteria. It lacks ZO-1 epitopes recognized by both monoclonal antibodies and a polyclonal serum to ZO-1, since it is not detectable in immunoblots of either whole cell extracts or ZO-1 immunoprecipitates. Also, it exhibits a peptide map different from that of ZO-1 on one-dimensional "Cleveland gels." Moreover, because the kinetics of appearance of newly synthesized 160-kDa polypeptide in anti-ZO-1 immunoprecipitates is much slower than that of ZO-1, its presence in immunoprecipitates cannot be simply explained by degradation of ZO-1 during cell lysis. Like ZO-1, the 160-kDa polypeptide seems to be a cytoplasmic peripheral membrane protein. It cannot be labeled by two different cell surface labeling reagents. It can be extracted from the membrane by high salt concentration in the absence of detergents. As expected for a protein complex, the 160-kDa polypeptide and ZO-1 turn over with similar kinetics. We propose that the 160-kDa polypeptide is a component of the tight junction.**

The tight junction, or zonula occludens, is an element of the epithelial junctional complex that is essential to the function of epithelial tissues (1–3). It seals the epithelial cells together at their apices to create the primary barrier to the diffusion of solutes through the paracellular pathway. The tight junction also contributes to the polarization of the epithelial cell surface because it forms a boundary between the apical and basolateral plasma membrane domains. The tight junction also seems to be a dynamic, regulated structure. Its permeability properties vary greatly among different epithelia (4) and even seem to be physiologically modulated within certain epithelia by exogenous stimuli (5).

Until recently, investigations of the structure and regulation of the tight junction have had to rely on ultrastructural methods. These studies, while informative, have resulted in conflicting models of tight junction structure and the changes underlying its regulation (6–10). An analysis of the structure of the tight junction at the molecular level will contribute significantly to the solution of these problems.

Two protein components of the tight junction have been described. ZO-1 is a 210- to 220-kDa peripheral membrane polypeptide tightly associated with the cytoplasmic surface of the tight junction (11). Cingulin is a 140-kDa protein localized to the region of the tight junction but not tightly associated with the membrane (12, 13). These are not likely to be the only polypeptide components of the tight junction. For example, it is often presumed that there must be one or more tight junction proteins expressed on the outer cell surface and/or spanning the plasma membrane (3, 14, 15).

The known tight junction polypeptides can now be used as tools to identify additional tight junction components. Because ZO-1 associates tightly with the plasma membrane at the tight junction, it was used in this study to try to identify novel ZO-1 binding proteins. We report the identification and preliminary characterization of a 160-kDa polypeptide that remains tightly associated with ZO-1 in anti-ZO-1 immunoprecipitates from detergent-solubilized Madin–Darby canine kidney (MDCK) cells.

MATERIALS AND METHODS

Cell Culture and Labeling. Strain II MDCK cells were grown in Eagle's minimal essential medium with 5% supplemented calf serum (16). Cells were metabolically labeled overnight with [^{35}S]methionine ([^{35}S]methionine/[^{35}S]cysteine, Tran^{35}S-label; ICN) at 0.5 mCi/ml (1 mCi = 37 MBq) in methionine-free growth medium containing 10% supplemented calf serum (4 ml of labeling medium per 75 cm^2 of cells). For shorter, pulse-labeling experiments, cells were first rinsed and incubated for 10 min in methionine-free, serum-free growth medium at 37°C and then labeled in the same medium with [^{35}S]methionine at 1 mCi/ml for 30 min. For a nonradioactive "chase" cells were incubated in complete growth medium containing 10 times the normal concentration of methionine.

Immunoprecipitation and Protein Analysis. All buffers for the extractions of cells and membranes contained the following protease inhibitors: phenylmethylsulfonylfluoride, 1 mM; iodoacetamide, 0.5 mM; pepstatin A, 1 μg/ml; leupeptin, 2 μg/ml; aprotinin, 4 μg/ml; antipain, 10 μg/ml; benzamidine, 50 μg/ml; soybean trypsin inhibitor, 10 μg/ml. Total SDS extracts of MDCK cells were prepared as described (17). For immunoprecipitations (18) with anti-ZO-1-Sepharose, cells were extracted with immunoprecipitation (IP) buffer [1% (vol/vol) Triton X-100/0.5% (wt/vol) sodium deoxycholate/0.2% (wt/vol) SDS/150 mM NaCl/10 mM Hepes, pH 7.4]. The R26.4C hybridoma producing the anti-ZO-1 monoclonal antibody (mAb) (19) was obtained from Bruce Stevenson (University of Alberta, Edmonton) and the mAb was purified from hybridoma culture supernatants on protein G-Sepharose (Pharmacia) according to the manufacturer's instructions. It was coupled to CNBr-activated Sepharose (Sigma) according to the instructions provided by Pharmacia. The nonimmune control antibody was one of several irrelevant mAbs specific for E-cadherin in *Xenopus laevis* (18), coupled

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Abbreviations: IP buffer, immunoprecipitation buffer; mAb, monoclonal antibody; PBS, phosphate-buffered saline (Eisen formulation).

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directly to Sepharose in the same way. Sample binding to concanavalin A (Con A)-Sepharose (Sigma) was done in the same way as the immunoprecipitations.

Immunoblotting was carried out as described (20) except that binding of the R26.4C anti-ZO-1 monoclonal antibody was detected using a biotin–avidin system: biotinylated-anti-rat IgG, followed by the reagents in the alkaline phophatase Vectastain ABC kit (Vector Laboratories). The rabbit anti-ZO-1 polyclonal antibody was obtained from James Anderson (Yale University School of Medicine). It had been raised against a fusion protein derived from a cDNA encoding human ZO-1 (21) and affinity-purified on the fusion protein.

All samples were analyzed by SDS/5% PAGE and fluorography (for ^{35}S) or autoradiography (for ^{125}I) as described (20). To quantify the radiolabel in individual polypeptides in immunoprecipitates, fluorographs were scanned with an LKB laser densitometer. Several exposures of the fluorographs were scanned to ensure that measurements were made over a linear range of film exposure. Data analysis and determination of half-lives were done using the Cricket Graph software for the Apple Macintosh.

Cell Surface Labeling, Peptide Mapping, and Membrane Extractions. To label proteins on all surfaces of MDCK cells, cells grown just to confluence in 75-cm^2 flasks were first incubated in phosphate-buffered saline, Eisen formulation (PBS), containing 2 mM EGTA at 37°C for ≈10 min to open the tight junctions. Lactoperoxidase-catalyzed cell surface iodination in PBS was performed as described (22). Cell surface biotinylation was carried out as described (23). Biotinylated proteins were detected with the Vectastain kit mentioned above.

Peptide mapping of the 160-kDa polypeptide and ZO-1 was performed by the method of Cleveland *et al.* (24). ^{35}S-labeled polypeptides were obtained by anti-ZO-1 immunoprecipitation and SDS/PAGE. Autoradiographs were used to locate the correct bands, which were then excised from the dried gel. Excised bands were digested with protease added to sample wells during the subsequent SDS/PAGE step.

To determine the nature of the association of the 160-kDa polypeptide with the membrane, a crude membrane fraction was prepared from [^{35}S]methionine-labeled MDCK cells as described (11), except for the homogenization step. Cells were first swollen in hypoosmotic buffer (10 mM Tris, pH 7.5) on ice and homogenized in the same buffer by five passages through a 25-gauge needle. The efficiency of homogenization was >90% of the cells, as determined by monitoring the presence of intact nuclei free of cytoplasm by trypan blue staining and light microscopy. Pelleted membranes were resuspended in a 10:1 vol of either PBS, IP buffer, or one of the following buffers (all containing the protease inhibitors): 0.5 M Tris·HCl, pH 7.8; 0.5 M KCl/10 mM Hepes, pH 7.5; 50 mM HCO_3^-, pH 10.5; 0.5 M KI/10 mM Hepes, pH 7.5. Membranes were then pelleted at 100,000 × *g* for 1 hr. The supernatant was diluted 5-fold with IP buffer lacking NaCl, to reduce the salt concentration, and immunoprecipitated as described above.

RESULTS

Before immunoprecipitation, ZO-1 must be extracted from cells under fairly harsh conditions. ZO-1 was not readily solubilized from MDCK cells by nonionic detergents in isotonic saline solutions, but it was effectively solubilized either by mixtures of nonionic detergents, deoxycholate, and SDS (IP buffer), or by nonionic detergents in high-salt solutions (data not shown). When ZO-1 was immunoprecipitated from mixed detergent extracts of [^{35}S]methionine-labeled MDCK cells with mAb R26.4C coupled directly to Sepharose, a second polypeptide, of ≈160 kDa, was always observed to coimmunoprecipitate (Fig. 1, lane a). The 160-kDa polypeptide never appeared in nonimmune control precipitations with irrelevant mAbs (lane b). This polypeptide is therefore a candidate for a protein that binds to ZO-1 under fairly stringent buffer conditions.

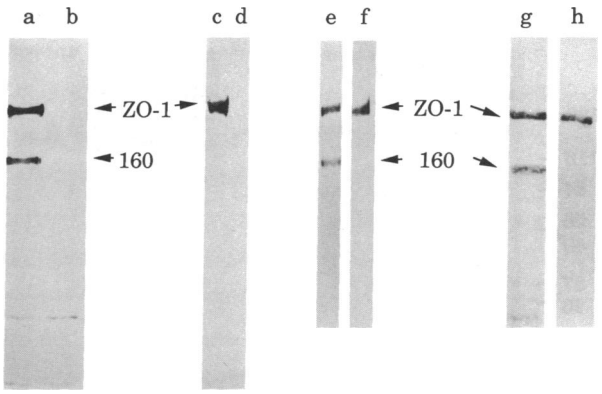

FIG. 1. Identification of a 160-kDa polypeptide that coimmunoprecipitates with ZO-1. Lanes a, e, and g, fluorographs of anti-ZO-1 (mAb) immunoprecipitates of [^{35}S]methionine-labeled MDCK cell proteins, obtained using mAb R26.4C coupled directly to Sepharose; lane b, nonimmune antibody control for the immunoprecipitation in lane a, using irrelevant mAb 5D3 coupled to Sepharose; lanes c and d, immunoblots of total SDS extracts of MDCK cells, probed with the anti-ZO-1 mAb and a nonimmune control antibody, respectively; lane f, an immunoblot of the immunoprecipitate shown in lane e, probed with the anti-ZO-1 mAb; lane h, an immunoblot of the immunoprecipitate shown in lane g, probed with a polyclonal antiserum raised to human ZO-1.

The stoichiometry of 160-kDa polypeptide to ZO-1 recovered in various immunoprecipitation experiments ranged from ≈0.5 to ≈1 when ^{35}S-labeled polypeptides were used for quantification. Similarly, a ratio of ≈0.5 was observed for Coomassie blue-stained polypeptides obtained in larger-scale immunopurifications of ZO-1 and the 160-kDa polypeptide (data not shown).

The 160-kDa polypeptide was itself not recognized by anti-ZO-1 antibodies. When total MDCK cell extracts were immunoblotted with the anti-ZO-1 mAb, only the 210-kDa ZO-1 polypeptide was detected (Fig. 1, lane c). Anti-ZO-1 immunoprecipitates that contained the ^{35}S-labeled 160-kDa polypeptide (lanes e and g) were also immunoblotted with anti-ZO-1 antibodies (lanes f and h). Neither the anti-ZO-1 mAb (lane f) nor a polyclonal serum with strong reactivity to ZO-1 (lane h) detected the 160-kDa polypeptide. These data indicate that the 160-kDa polypeptide does not contain epitopes recognized by the anti-ZO-1 antibodies. Presumably it coimmunoprecipitates with ZO-1 because it is tightly bound to ZO-1 under the conditions of the experiment.

Peptide mapping was performed to determine whether ZO-1 and the 160-kDa polypeptide might be distinct molecules. Their peptide maps on one-dimensional "Cleveland gels" differed in several respects (Fig. 2). First, ZO-1 was more labile than the 160-kDa polypeptide to digestion with either V8 protease or trypsin. Second, the fragments that were produced from the 160-kDa polypeptide by higher concentrations of the two proteases were not the same size as the major products of ZO-1 digestion. Incomplete digestion of the 210-kDa ZO-1 polypeptide by either V8 protease or trypsin did not generate a major polypeptide fragment with the mobility of the 160-kDa polypeptide. Therefore, intentional proteolysis of ZO-1 *in vitro* did not result in a degradation product with the size of the 160-kDa polypeptide. These differing peptide maps are not consistent with the simple notion that the 160-kDa polypeptide is a degradation product of ZO-1 but, rather, support the hypothesis that the 160-kDa polypeptide is biochemically distinct from ZO-1.

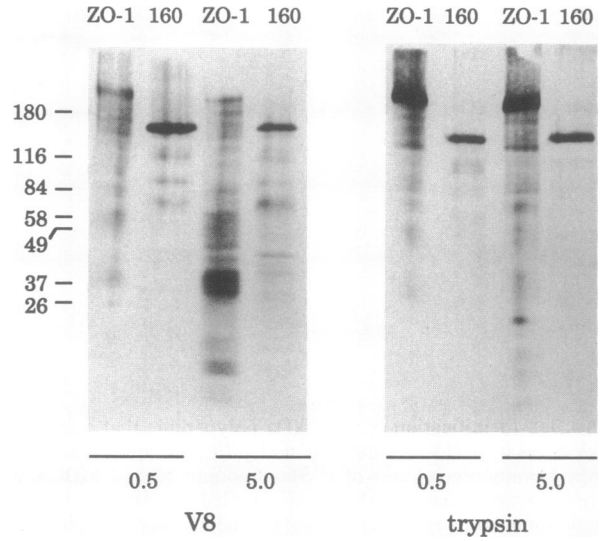

FIG. 2. Cleveland peptide maps of ZO-1 compared with the 160-kDa polypeptide. ZO-1 (ZO-1) and the 160-kDa polypeptide (160) were coimmunoprecipitated from [^{35}S]methionine-labeled MDCK cells and separated by SDS/PAGE. The bands were excised from the gel and individually digested with either *Staphylococcus aureus* V8 protease (0.5 or 5.0 μg/ml) or trypsin (0.5 or 5.0 μg/ml) and then analyzed by SDS/PAGE and fluorography. Dashes at left represent the migration positions of molecular size (kDa) standards, indicated at left.

To determine when ZO-1 and the 160-kDa polypeptide become associated in the cell, pulse–chase labeling experiments were carried out (Fig. 3). Immediately after a 30-min pulse with [^{35}S]methionine, primarily ZO-1, but little of the 160-kDa polypeptide, appeared in the immunoprecipitates (Fig. 3, 0-hr lanes). The labeled 160-kDa polypeptide was chased into an immunoprecipitable pool more slowly (2-, 4-, and 8-hr chase lanes). Significant incorporation of the labeled 160-kDa polypeptide occurred after ≈2 hr of chase and accumulated to a plateau at 4–8 hr of chase. Pulse–chase labeling over a shorter interval indicated that significant labeling of the 160-kDa polypeptide did not occur until after 1 hr of chase (data not shown). The lack of appearance of the 160-kDa polypeptide after the initial pulse-labeling period indicates that its coimmunoprecipitation with ZO-1 does not result from degradation of ZO-1 or artifactual binding during its isolation from the cell. Its chase into an immunoprecipitable pool over time probably reflects the kinetics of its intracellular association with ZO-1 (see *Discussion*).

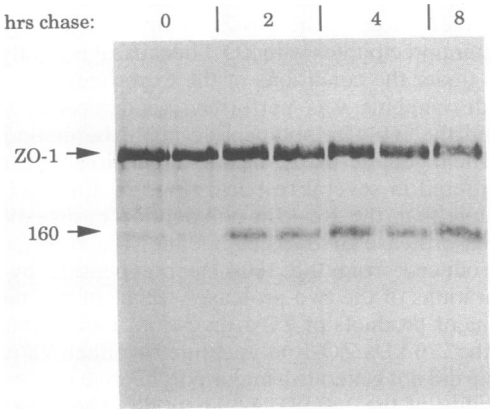

FIG. 3. Pulse–chase analysis of ZO-1/160-kDa polypeptide coimmunoprecipitates. MDCK cells were pulse-labeled with [^{35}S]methionine for 30 min and then incubated with unlabeled methionine for the times shown. Solubilized proteins were then immunoprecipitated with the anti-ZO-1 mAb, separated by SDS/PAGE, and detected by fluorography.

The rates of turnover of the 160-kDa polypeptide and ZO-1 in MDCK cells were also compared (Fig. 4). After long-term labeling with [^{35}S]methionine, cells were incubated in unlabeled medium for increasing periods of time, and the label remaining in each of the two polypeptides in the ZO-1 immunoprecipitates was determined. Although the absolute turnover rates for the two polypeptides varied from experiment to experiment depending on the culture conditions (compare Fig. 4 *A* and *B*), ZO-1 and the 160-kDa polypeptide always exhibited similar half-lives within a single experiment. In the experiment shown in Fig. 4*A* the half-lives for ZO-1 and the 160-kDa polypeptide were 18 hr and 19 hr, respectively, and in that shown in Fig. 4*B* they were 9.4 hr and 8.7 hr, respectively. Their similar rates of turnover, although possibly coincidental, are consistent with their existence as a stable complex in the cell that turns over as a unit.

Because ZO-1 is closely associated with the membrane of the tight junction, it was of great interest to determine whether the 160-kDa polypeptide might be a transmembrane tight junction protein. If the 160-kDa polypeptide is a transmembrane protein it should be exposed at the cell surface. We determined, therefore, whether the 160-kDa polypeptide was accessible to two different types of cell surface labeling. The extracellular surfaces of the tight junctions were first made accessible by incubating MDCK cells briefly in Ca^{2+}-free medium. Proteins on the cell surfaces were then labeled with ^{125}I by the lactoperoxidase-catalyzed reaction, and ZO-1 was immunoprecipitated (Fig. 5*A*). Neither the 160-kDa polypeptide, nor ZO-1, nor any other specifically immuno-

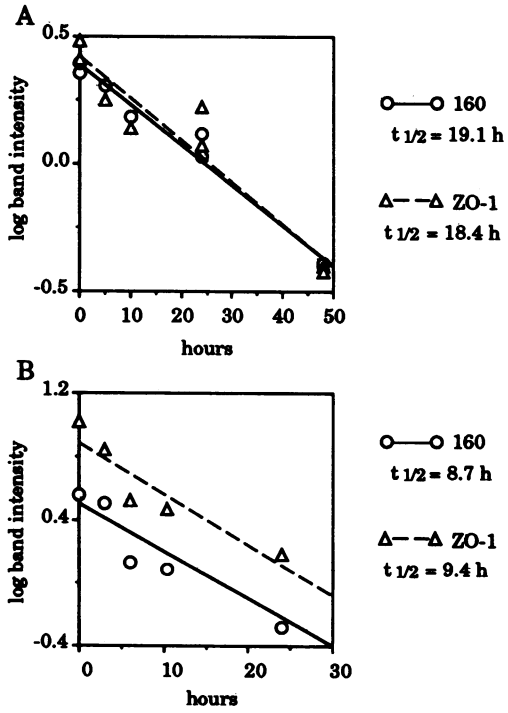

FIG. 4. Metabolic turnover of ZO-1 and the coimmunoprecipitated 160-kDa polypeptide in MDCK cells. MDCK cells were labeled for 16 hr with [^{35}S]methionine and then incubated with unlabeled methionine for various lengths of time. Anti-ZO-1 immunoprecipitates were separated by SDS/PAGE, and the resulting fluorographs were scanned by densitometry to determine the relative label remaining in each of the two polypeptides. (*A*) Confluent MDCK cells. Measured half-lives are 18.4 hr and 19.1 hr for ZO-1 and the 160-kDa polypeptide, respectively. (*B*) Subconfluent MDCK cells. Measured half-lives are 9.4 hr and 8.7 hr for ZO-1 and the 160-kDa polypeptide, respectively.

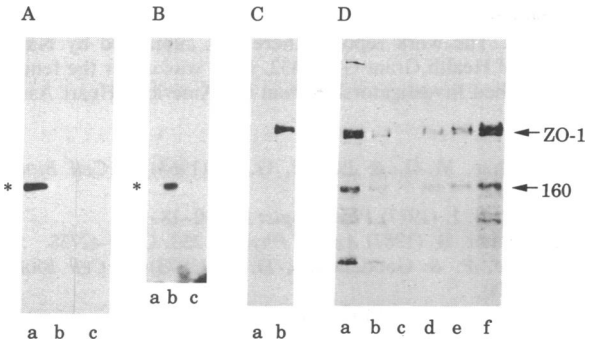

FIG. 5. Nature of the association of the 160-kDa polypeptide and ZO-1 with the plasma membrane. (*A*) Cell surface iodination. MDCK cells were surface-labeled by lactoperoxidase-catalyzed iodination. Solubilized proteins were immunoprecipitated and analyzed by SDS/PAGE and autoradiography. Lane a, anti-E-cadherin; lane b, anti-ZO-1 mAb; lane c, nonimmune antibody. Asterisk indicates position of E-cadherin. (*B*) Cell surface biotinylation. MDCK cells were surface-labeled with NHS-LC-biotin (Pierce). Immunoprecipitates were separated by SDS/PAGE and labeled proteins were detected by Western blotting with alkaline phosphatase-conjugated avidin. Lane a, anti-ZO-1 mAb; lane b, anti-E-cadherin; lane c, nonimmune antibody. (*C*) Lack of binding of the ZO-1/160-kDa polypeptide complex to Con A-Sepharose. Detergent extracts of MDCK cells were incubated with Con A-Sepharose. The bound fraction (lane a) and the unbound fraction (lane b) were analyzed by SDS/PAGE and immunoblotting with anti-ZO-1 mAb. (*D*) Extraction of the 160-kDa polypeptide and ZO-1 from MDCK cell plasma membranes by various solutions. A crude membrane fraction of [^{35}S]methionine-labeled MDCK cells was incubated in one of the solutions listed below. The solubilized proteins in the supernatants were diluted to reduce the salt concentration, immunoprecipitated with anti-ZO-1, and analyzed by SDS/PAGE and fluorography. Lane a, 0.5 M Tris buffer; lane b, 0.5 M KCl buffer; lane c, PBS; lane d, detergent-containing IP buffer; lane e, pH 10.5 carbonate buffer; lane f, 0.5 M KI buffer.

precipitated polypeptides were detected (lane b). The cell surface adhesion protein E-cadherin was readily detected after its immunoprecipitation from the same samples (lane a), demonstrating the efficacy of the cell surface labeling reaction.

Lactoperoxidase-catalyzed iodination labels only exposed tyrosine residues of surface proteins. Therefore, the biotinylation reaction, which modifies exposed primary amino groups, was also used for cell surface labeling (Fig. 5*B*). In the great majority of experiments the biotin moiety could not be detected in any polypeptide immunoprecipitated with anti-ZO-1 antibodies (lane a).† Biotinylated E-cadherin was easily detected (lane b), again demonstrating that the surface labeling was successful. Therefore, the 160-kDa polypeptide, like ZO-1, was not accessible to surface labeling. The lack of labeling suggests that the 160-kDa polypeptide is contained entirely within the cytoplasm. It is not possible to exclude, however, that the negative result arose from some unknown technical problem.

Because many cell surface glycoproteins bind to the lectin Con A (25), we asked whether ZO-1 was extracted from MDCK cells in a complex with a Con A-binding polypeptide, which could be either the 160-kDa polypeptide or any as yet unidentified polypeptide. ZO-1 was not detectable in the protein fraction bound to Con A (Fig. 5*C*, lane a) but rather was recovered entirely in the unbound fraction (lane b). Therefore, ZO-1 extracted from MDCK cells under these harsh conditions is not part of a protein complex containing significant amounts of Con A-binding carbohydrates. Presumably, the 160-kDa polypeptide, which we know is bound to ZO-1 under these conditions, also lacks Con A-binding oligosaccharide chains.

If the 160-kDa polypeptide is a peripheral membrane protein like ZO-1, it should be extractable from the membrane by high-salt buffers in the absence of detergents. A crude membrane fraction prepared from [^{35}S]methionine-labeled MDCK cells was extracted under various conditions (Fig. 5*D*). High Tris (lane a), high KCl (lane b), pH 10.5 (lane e), and KI (lane f) all released both ZO-1 and the 160-kDa polypeptide from the membrane into the supernatant. In contrast, isotonic PBS did not release them from the membranes (lane c). Therefore, the 160-kDa polypeptide, like ZO-1, does not require detergents for its solubilization from the membrane but rather is attached to the membrane in a salt- and pH-labile linkage.

DISCUSSION

In this paper we report the identification of a 160-kDa polypeptide that coimmunoprecipitates with ZO-1 solubilized from MDCK cells. The primary evidence that the 160-kDa polypeptide is an entity distinct from ZO-1 is that it seems not to harbor the major ZO-1 epitopes. Neither the mAb R26.4C nor the polyclonal anti-ZO-1 antiserum, which reacts even more avidly with MDCK ZO-1 (data not shown), recognized the 160-kDa polypeptide on immunoblots. Because the 160-kDa polypeptide does not bind directly to the anti-ZO-1 antibodies, it must coimmunoprecipitate with ZO-1 because it is associated with ZO-1 in a protein complex.

The association between ZO-1 and the 160-kDa polypeptide seems to be due to a specific intracellular interaction rather than an artifactual association caused during cell lysis. The polypeptides formed a very stable protein–protein interaction that survived the relatively harsh buffer conditions required to extract ZO-1 from the cell. Also, they remained associated even when they were extracted from the membrane by high salt or high pH. Furthermore, the 160-kDa polypeptide did not appear in immunoprecipitates of cells labeled with a short pulse of [^{35}S]methionine. Therefore, the appearance of the 160-kDa polypeptide in immunoprecipitates results from a time-dependent intracellular event, rather than an artifactual association induced during sample preparation.

It is extremely unlikely that the 160-kDa polypeptide is simply a proteolytic degradation product of ZO-1 that remains associated with intact ZO-1. Peptide mapping experiments indicated that the 160-kDa polypeptide has an amino acid sequence distinct from ZO-1. In fact, intentional proteolysis of ZO-1 did not yield a digestion product the size of the 160-kDa polypeptide, a result that would have been expected if degradation of ZO-1 to the 160-kDa polypeptide were to occur at an especially susceptible site. Also, the absence of the 160-kDa polypeptide in immunoprecipitates after a short pulse-labeling with [^{35}S]methionine demonstrated that its presence in immunoprecipitates did not result from proteolysis during sample preparation. Again, the appearance of the 160-kDa polypeptide in immunoprecipitates must result from a time-dependent intracellular event.

The appearance of the 160-kDa polypeptide with increasing times of chase is very unlikely to be due to a normal physiological conversion of ZO-1 to the 160-kDa polypeptide. Again, the lack of 160-kDa recognition by anti-ZO-1 in immunoblots and the differing peptide maps are not consistent with the 160-kDa polypeptide being derived from ZO-1.

†Occasionally, biotinylated polypeptides that migrated with ZO-1 and the 160-kDa polypeptide were immunoprecipitated (data not shown). In these cases, however, the intracellular cytoskeletal proteins vinculin and α-actinin were also labeled. Presumably, ZO-1, the cytoskeletal proteins, and the 160-kDa polypeptide were labeled in occasional experiments because the plasma membranes became partially permeable during the opening of the tight junctions by Ca^{2+} depletion.

Moreover, the initial rate of appearance of the 160-kDa polypeptide, with a half-time of 1.8 hr (data not shown), was considerably faster than the rate of turnover of ZO-1. Therefore, the time of incorporation of pulse-labeled 160-kDa polypeptide into the immunoprecipitable pool probably reflects the kinetics of its intracellular association with ZO-1. These kinetics could be due either to the rate of its intracellular assembly into a complex with ZO-1 or to the existence of a large precursor pool of unassembled 160-kDa polypeptide.

ZO-1 and the 160-kDa polypeptide may form a very stable association within the cell. They exhibited very similar rates of metabolic turnover in the cell and remained associated *in vitro* even in fairly harsh solutions. Since all of the ZO-1 in the MDCK cell seems to be associated with the tight junction (11), any of the 160-kDa polypeptide stably bound to it would presumably be associated with the tight junction. However, until specific antibodies to the 160-kDa polypeptide are available, it will not be possible to know whether it is exclusively associated with the tight junction, or exists in other intracellular location(s) as well.

We propose that the 160-kDa polypeptide is a component of the epithelial tight junction. To our knowledge there are no known proteins of similar sizes that are candidates for being tight junction-associated proteins. Cingulin, the other protein known to be associated with the tight junction, has a molecular mass of 140 kDa as determined by SDS/PAGE (12). Also, antibodies to cingulin do not recognize the 160-kDa polypeptide on immunoblots (S. Citi and B.G., unpublished observations). The major known proteins associated with the nearby zonula adherens junction, α-actinin, vinculin, radixin, tenuin, and actin, have distinctly different molecular sizes (26–28). To demonstrate with certainty that the 160-kDa polypeptide is a novel polypeptide and that it is a bona fide tight junction component in epithelial cells, it will be necessary to raise antibodies specific for it and to clone the cDNA encoding it.

We tentatively propose the name "ZO-2" (for zonula occludens-2) for the 160-kDa polypeptide. Once specific reagents for ZO-2 become available, it will be possible to explore further its molecular nature, its interaction with ZO-1, its intracellular and tissue distributions, and its function in the tight junction. Although ZO-2 is not the transmembrane component of the tight junction, its characterization may contribute to determining the molecular structure of the tight junction and perhaps will be important for understanding tight junction function.

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