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The tight junction protein ZO-1 is homologous to the *Drosophila* discs-large tumor suppressor protein of septate junctions

(zonula occludens/epithelia/endothelia/intercellular junction)

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ABSTRACT **Tight junctions form an intercellular barrier between epithelial cells, serve to separate tissue compartments, and maintain cellular polarity. Paracellular sealing properties vary among cell types and are regulated by undefined mechanisms. Sequence of the full-length cDNA for human ZO-1, the first identified tight junction component, predicts a protein of 1736 aa. The N-terminal 793 aa are homologous to the product of the lethal(1)discs-large-1 (*dlg*) tumor suppressor gene of *Drosophila*, located in septate junctions, and to a 95-kDa protein located in the postsynaptic densities of rat brain, PSD-95. All three proteins contain both a src homology region 3 (SH3 domain), previously identified in membrane proteins involved in signal transduction, and a region homologous to guanylate kinase. ZO-1 contains an additional 943-aa C-terminal domain that is proline-rich (14.1%) and contains an alternatively spliced domain, whose expression was previously shown to correlate with variable properties of tight junctions. *dlg* mutations result in loss of apical-basolateral epithelial cell polarity and in neoplastic growth. These results suggest a protein family specialized for signal transduction on the cytoplasmic surface of intercellular junctions. These results also provide biochemical evidence for similarity between invertebrate septate and vertebrate tight junctions. The C-terminal domain of ZO-1, and its alternatively spliced region, appears to confer variable properties unique to tight junctions.**

Tight junctions, zonula occludentes, form regulated intercellular barriers between both epithelial and endothelial cells and serve to separate tissue compartments by limiting ability of solutes, water, and immune cells to traverse the paracellular space. Tight junctions also separate biochemically distinct apical and basolateral plasma membrane surfaces contributing to maintenance of cellular polarity. The paracellular barrier of tight junctions varies among cell types with respect to electrical resistance and charge and size of molecules permitted to pass through the junction. These properties are hormonally regulated *in vivo* and in cultured epithelial and endothelial cells (1, 2). The ability of junctions to assemble and disassemble in cultured cells is influenced by protein kinases A and C as well as guanine nucleotide binding (G)-protein pathways (3, 4); however, the specific sites of regulation are undefined.

In cross-sectional images, the tight junction is composed of several points, or "kisses," at which the membranes of adjacent cells come into close contact. Contacts are presumed to be formed by rows of transmembrane proteins forming homotypic associations with those on adjacent cells to create a continuous seal around each cell (1, 5). Epithelial cells of invertebrates contain a barrier-forming junction called the septate junction (6), which is ultrastructurally distinct from tight junctions. Until the present report, to our knowledge, no biochemical similarities between septate and tight junctions had been identified. Mutations resulting in absence of the dlg protein, which is normally located on or near the cytoplasmic surface of *Drosophila* septate junctions, lead to loss of cellular polarity and neoplastic growth of epithelial cells within larval imaginal discs (7, 8). Although tight junctions are aberrantly organized in mammalian carcinomas (9), they are not known to directly regulate cell proliferation.

ZO-1, the first identified protein of tight junctions, is an ≈220-kDa peripheral membrane phosphoprotein expressed in tight junctions of both epithelial and endothelial cells (10, 11). Ultrastructural immunogold localization shows ZO-1 located on the cytoplasmic membrane surface precisely at sites of cell–cell contact (10). ZO-1 has also been localized to the highly modified tight junctions of glomerular epithelial cells (12) and to the cell–cell contacts of several cultured nonepithelial cells, including brain astrocytes (13). We previously reported the partial cDNA sequence of both rat and human ZO-1 including a 240-bp region that is alternatively spliced (11, 14). Two protein isoforms are generated that differ by an 80-aa domain. Immunolocalization studies suggest junctions express one or the other isoform and expression of the shorter isoform correlates with greater structural plasticity (11). Several other tight junction proteins have been identified, including cingulin (15), ZO-2 (16), and the 7H6 antigen (17). The function of all these proteins remains unknown. Although junctional permeability and structural organization are regulated (1, 2), a role for tight junctions in transducing intercellular signals has not been previously proposed.

We now report the full-length cDNA sequence of human ZO-1‡ and its homology to the product of the lethal(*1*)discs-large-1 (*dlg*) tumor suppressor gene of *Drosophila* and a postsynaptic density (PSD) protein from rat brain (PSD-95; ref. 18). We suggest ZO-1 is a member of a family of proteins involved in signal transduction at specialized cell–cell junctions and that ZO-1 contains additional sequences responsible for defining specific properties of tight junctions.

MATERIALS AND METHODS

Molecular Techniques. Techniques used for library screening, cloning, and DNA sequencing have been described (14). All libraries were constructed in the λ ZAP vector (Strata-

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Abbreviations: GK, guanylate kinase; G protein, guanine nucleotide binding protein; SH3, *src* oncogene homology region 3; PSD, postsynaptic density.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. L14837).

gene). Hybridization probes were generated by labeling purified cDNA fragments by the random-priming technique (Boehringer Mannheim) using [α-^{32}P]dCTP (Amersham) and blots were hybridized and washed as described (14). Priming was with either the T7 and T3 sites of pBluescript (Promega) or ZO-1-specific primers. Methods for RNA isolation and Northern blot analysis have been described (19). Southern blot analysis of genomic DNA from human peripheral blood leukocytes was performed using standard techniques (20) and was washed for two 15-min periods at 50°C in 0.1% SDS/15 mM NaCl/1.5 mM sodium citrate, pH 7.0.

Sequence Analysis. DNA and protein sequences were compared by the BESTFIT and PILEUP algorithms using the University of Wisconsin Genetics Computer Group Software Package (21). Sequences were obtained from the GenBank (Release No. 74) and the Protein Identification Resource (Release No. 34) data bases.

RESULTS AND DISCUSSION

Cloning of the Human ZO-1 cDNA. The human cDNA, B11 (14), was used as the initial probe and multiple overlapping clones representing the full-length cDNA were obtained by screening four separate libraries constructed from RNA isolated from either human liver or the human HepG2 hepatoma cell line (Stratagene) (Fig. 1). Clones encoding the 5′-most sequence were obtained from two libraries (Fig. 1, libraries 1 and 2) made by priming poly(A)$^+$ RNA from human liver with specific antisense ZO-1 primers after methylmercury denaturation (Clontech). The 5′ untranslated region of the isolated cDNA contains several G+C-rich regions, is 66% G+C overall, and is presumably highly structured. High G+C content in long 5′ untranslated regions has been noted in mRNAs whose expression is highly regulated, including those encoding oncogenes, membrane kinases, and other proteins involved in cellular signal transduction (22). Northern blot analysis detects a major 7.9-kb mRNA species encoding ZO-1 in all tissues tested, consistent with the identified cDNA of 7888 bp (see Fig. 3*A*).

An open reading frame is present from positions 1227 to 6434. We assign the translation start site based on a good match with the eukaryotic initiation consensus sequence (23); an alternative initiation site is present in frame at position 1191 but contains a weak consensus. The deduced amino acid sequence of 1736 residues predicts a protein of 194.7 kDa (Fig. 2). This size is 12% smaller than the apparent molecular mass determined by SDS/PAGE. The high proline content (9.7%) may be responsible for this anomalous electrophoretic migration, as is characteristic of other proline-rich proteins (24). Identification of the proper reading frame is supported

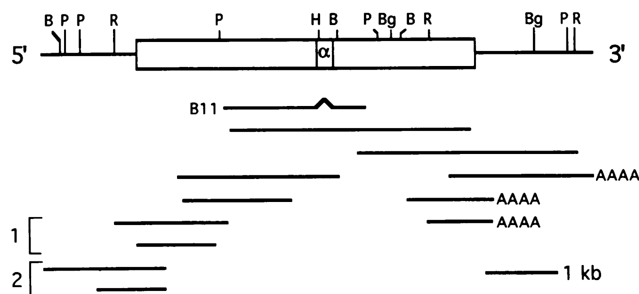

FIG. 1. Map of human ZO-1 cDNA. Locations of *Bam*HI (B), *Pst* I (P), *Eco*RI (R), *Bgl* II (Bg), and *Hin*dIII (H) sites are shown. Open reading frame is boxed and includes the alternatively spliced region, α. A limited set of all cDNAs sequenced is presented, including the initial human cDNA, B11 (Willott), and clones representing two polyadenylylation sites. Primers used to construct library 1 were positions 2386–2405 and positions 2649–2660 and library 2 were positions 1751–1770; two products from each are shown.

by the production of antibodies that exclusively recognize ZO-1 against two recombinant fusion proteins and a peptide predicted by this open reading frame (11, 14). Three clones were isolated that demonstrate alternative splicing between positions 3953 and 4194 (see Fig. 2). Polyadenylylated clones were isolated that terminate at position 6463 or 7888, the first preceded by an A+T-rich region and the second preceded by two polyadenylylation consensus sequences (Fig. 2). The longer polyadenylylation acceptor site is more prevalent based on mRNA size detected by Northern blot analysis (Fig. 3*A*).

Genomic Southern blot analysis was performed on DNA isolated from human peripheral blood leukocytes (Fig. 3*B*). After digestion with one of four restriction endonucleases and washing at moderate stringency, hybridization to single bands was detected. This is consistent with ZO-1 being encoded by a single gene.

Sequence Homologies. The N-terminal 793 residues of ZO-1 form a basic (pI = 9.3) 89.4-kDa domain that is homologous to the dlg tumor suppressor of *Drosophila* (26% identical, 47% similar; ref. 8), PSD-95 from rat brain synapses (31% identical, 55% similar; ref. 24), and the human erythrocyte membrane protein p55 (24% identical, 47% similar; ref. 18). dlg and PSD-95 appear more closely related to each other (58% identical, 76% similar) than to either ZO-1 or p55 (Fig. 4).

The dlg product is located at the apical-lateral membrane boundary of epithelial cells in the adult fly, presumably in septate junctions (8), comparable to the localization of ZO-1 in tight junctions. dlg is present in the early blastoderm and associates with the plasma membrane preceding septate junction formation at the time of dorsal closure. Mutations of the dlg protein result in loss of normal apical-basolateral polarity and intercellular adhesion of epithelial cells within imaginal discs. Cells become cuboidal, develop intercellular gaps, and during an abnormally prolonged larvae stage, proliferate to form solid tumor. Some mutant alleles of *dlg* permit the formation of septate junctions but neoplastic growth still occurs (7, 8). These results suggest *dlg* is involved in signal transduction necessary for proliferation control in mitotically active tissues. The PSD-95 protein has been identified as a component of the PSD of rat brain, a site of cell–cell contact specialized for information transfer. Although neurons do not undergo mitosis, the homology between dlg and PSD-95 has led to speculation that PSD-95 is involved in synaptic signaling or organization (18). p55 is a palmitoylated protein of the human erythrocyte membrane that copurifies with the actin-bundling protein dematin and a serine kinase (25). The function of p55 is unknown, but based on homology with dlg, a role in differentiation of the erythroid lineage has been proposed (26).

All four proteins contain domains of homology with the *src* oncogene homology region 3 (SH3) as defined by Musacchio *et al.* (27). This ≈60-residue motif was originally identified in the noncatalytic region of the src family of protein tyrosine kinases and is now recognized in numerous signaling and cytoskeletal proteins (27, 28). Emerging evidence suggests SH3 domains bind to a variety of different proteins that regulate activity of small GTP-binding proteins by controlling the ratio of bound GTP to GDP (e.g., through accelerating hydrolysis of bound GTP to GDP or stimulating or inhibiting release of bound GDP). For example, SH3 domain of the protooncogene c-*abl* binds to a member of the GTPase-activating protein (GAP) rho family. Mutation of the SH3 domain activates the c-*abl* transforming ability (29). GAP rho catalyzes hydrolysis of GTP to GDP bound to small GTP-binding proteins, which is expected to inactivate ras-like proteins. The SH3 domain of the dlg protein has been shown to be most closely homologous to that of the oncogene v-*crk*

FIG. 2. Complete cDNA and deduced amino acid sequence of human ZO-1. Amino acid residues are numbered on the right; nucleic acid positions are numbered on the left; the amino acid sequence is above the nucleic acid sequence. Potential upstream initiation codon at position 1191 is boxed. Two 90-aa repeats of unknown function are identified with a thin dashed line (aa 11–97 and aa 411–491); SH3 domain, with a thick solid line (aa 508–569); guanylate kinase (GK) domain, with a thin solid line (aa 632–782); acid domain, with a thick dashed line (aa 806–883). The alternatively spliced region is boxed (aa 910–989). Polyadenylylation acceptor signals are boxed beginning at residues 7862 and 7876.

(8). This sequence is included in Fig. 5*A* and Table 1 for comparison.

ZO-1 like dlg, PSD-95, and p55 also contains a region of homology to the yeast GK, as shown in Fig. 5*B* and Table 2. GK catalyzes the conversion of 5′-GMP + ATP → GDP + ADP (30). GK activity of the dlg product is proposed to elevate GDP relative to GTP within the septate junction and inactivate local G proteins. Mutations eliminating this activ-

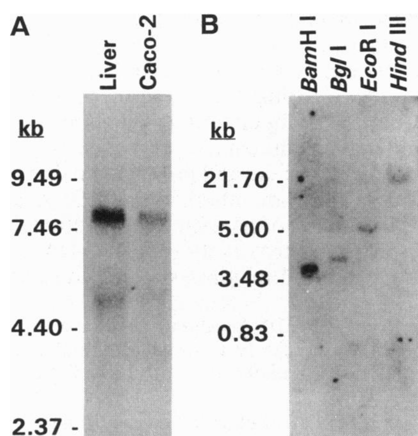

FIG. 3. Analysis of human ZO-1 gene and mRNA. (*A*) Northern blot analysis of human total RNA probed with a radiolabeled cDNA fragment corresponding to positions 2717–6123 of ZO-1. A predominant band of ≈7.9 kb and a minor hybridization to the 28S band of rRNA (5.2 kb) are detected in liver and Caco-2 cultured enterocytes. Size markers are designated in kilobases (GIBCO/BRL RNA ladder). (*B*) Southern blot analysis of human genomic DNA (20 μg per lane) digested with the indicated restriction endonucleases and probed with a radiolabeled 469-bp cDNA fragment of ZO-1 (residues 4583–5052). Size standards indicated to left were determined from a *Hin*dIII/*Eco*RI digest of λ DNA.

ity would elevate GTP/GDP levels, activate G proteins, and stimulate proliferation (8, 26). However, it is not clear whether ZO-1, dlg, or PSD-95 can function as active kinases. All adenylate kinases and GKs contain conserved residues forming an "anion binding hole" for the PO_4 groups of ATP; these are residues Gly-8 to Ser-15 of yeast GK (Fig. 5, ref. 31). Three of the 8 residues in this region are deleted in ZO-1, dlg, and PSD-95, although p55 maintains these residues. The crystal structure of yeast GK with bound GMP has been solved (31). GMP binds to yeast GK through hydrogen bonds at residues Arg-38, Arg-41, Tyr-50, and Tyr-78 and directly at Ser-34, Glu-69, Ser-80, and Asp-100 (Fig. 5*B*). This region is poorly conserved in ZO-1, with substitutions or a deletion at four sites and a conspicuous 28-residue deletion that eliminates three additional GMP binding sites (Fig. 5). Thus, ZO-1 is very unlikely to have GK activity. Conservation of residues required for GMP binding is better in dlg, PSD-95, and p55; however, direct evidence for GK activity in these proteins is lacking. Alleles of dlg that delete only the C-terminal GK domain still induce the mutant phenotype, strongly suggesting an important, although undefined, activity for this domain. The structure of GK is similar to that of small GTP-binding proteins (31). Thus an alternative hypothesis is that the GK homology domains of this family interact with signaling pathways via G-protein-binding proteins (e.g., GTPase-activating proteins) and not by altering GDP/GTP ratios.

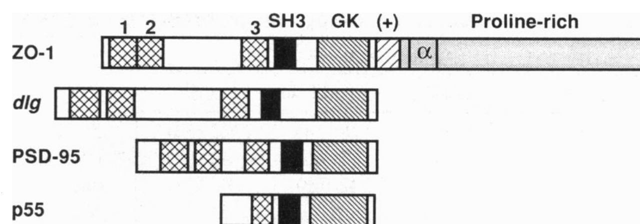

FIG. 4. Schematic comparison of structural domains within ZO-1, dlg, PSD-95, and p55 proteins. Repeated domains of unknown function are numbered 1, 2, and 3. SH3, GK homology region, acidic domain (+), alternatively sliced domain (α), and proline-rich domains are noted.

Like dlg and PSD-95, the N-terminal region of ZO-1 contains a repeating 90-aa motif whose function is unknown. Both dlg and PSD-95 contain three repeats. Repeats 1, 2, and 3 are, respectively, more similar between dlg and PSD-95 than within each protein, suggesting a common evolutionary origin for the entire colinear set of protein domains. Although ZO-1 also has three repeats, repeat 2 is not well conserved. Comparison of ZO-1 and dlg shows that the first repeat is 44% identical (68% similar) and the third repeat is 42% identical (70% similar). However, the second repeat is only 22% identical (40% similar). These repeats have been arbitrarily referred as GLGF repeats (18), although the GLGF motif is not conserved in either ZO-1 or p55. p55 contains a single repeat, most homologous to repeat 3.

A SH3 Domains

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              10         20         30         40         50         60        69
ZO1   YIRTHFEYEK ESP....... YGLSFNKGEV FRAVDTLYNG KLGSWLAIRI GKNHKEVERG IIPNKNRAE
PSD   YIRALFDYDK TKDCGFLSQ. .ALSFRFGDV LHVID...AG DEEWWQARRV HSDSETDDIG FIPSKRRVE
DLG   YVRALFDYDP NRDDGLPSR. .GLPFKHGDI LHVTN...AS DDEWWQARRV LGDNEDEQIG IVPSKRRWE
P55   FMRAQFDYDP KKDNLIPCKE AGLKFATGDI IQIIN...KD DSNWWQGR.. VEGSSKESAG LIPSPELQE
CRK   YVRALFDFKG NDDGD..... ..LPFKKGDI LKIRD...KP EEQWWNA... ..EDMDGKRG MIPV.....
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B Guanylate Kinase Domains

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              10         20         30         40         50         60         70
ZO1   LRPVTIFGPI A...DVAREK LAREEPDIYQ IAKS....EP RDAGTDQRSS GYI....... ..........
PSD   ARPIIILGP. ..TKDRANDD LLSEFPDKFG SCVPHTTRPK REYEIDGRDY HFVSSREKME KDIQAHKFIE
DLG   TRPVIILGP. ..LKDRINDD LISEYPDKFG SCVPHTTRPK REYEVDGRDY HFVSSREQME RDIQNHLFIE
P55   RKTLVLIGAS GVGRSHIKNA LLSQNPEKFV YPVPYTTRPP RKSEEDGKEY HFIST.EEMT RNISANEFLE
GK    SRPIVISGPS GTGKSTLLKK LFAEYPDSFG FSVSSTTRTP RAGEVNGKDY NFVSV.DEFK SMIKNNEFIE
                                          ●   *   *        *                     ●

              80         90        100        110        120        130        140
ZO1   .......... .RLHTIKQII DQDKHALLDV TPNAVDRLN. YAQWYPIVVF LNPDSKQGVK TMRMRLCPES
PSD   AGQYNSHLYG TSVQSVREVA EQGKHCILDV SANAVRRLQA .AHLHPIAIF IRPRSLENVL EINKRITEE.
DLG   AGQYNDNLYG TSVASVREVA EKGKHCILDV SGNAIKRLQV .AQLYPVAVF IKPKSVDSVM EMNRRMTEE.
P55   FGSYQGNMFG TKFETVHQIH KQNKIAILDI EPQTLKIVRT .AELSPFIVF IAPTD..... ...QGTQTE.
GK    WAQFSGNYYG STVASVKQVS KSGKTCILDI DMQGVKSVKA IPELNARFLF IAPPSVEDLK KRLEGRGTE.
              *  ●                     ●

             150        160        170        180        190
ZO1   RKSARKLYER SHKLAKNNHH LFTTTINLNS MNDGW...YG ALKEAVQQQQ
PSD   ..QARKAFDR ATKLEQEFT. ...ECFSAIV EGDSFEEIYH KVKRVIEDLS
DLG   ..QAKKTYER AIKMEQEFG. ...EYFTGVV QGDTIEEIYS KVKSMIWSQS
P55   ..ALQQLQKD SEAIRSQYAH ....YFDLSL VNNGVDETLK KLQE.AFDQA
GK    ..TEESINKR LSAAQAELAY AETGAHDKVI VNDDLDKAYK ELKDFIFAEK
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FIG. 5. Amino acid sequence comparisons of ZO-1, PSD-95, dlg, and p55. (*A*) Putative SH3 domain in ZO-1 (aa 508–569) is compared with those of PSD-95 (aa 432–495), dlg (aa 604–667), p55 (aa 162–225), and v-crk (aa 372–420). Residues identical to ZO-1 are shaded. (*B*) GK homology domain of ZO-1 (aa 632–782) is compared to PSD-95 (aa 534–712), dlg (aa 770–948), p55 (aa 282–454), and yeast GK (aa 1–186). Residues identical to ZO-1 are shaded. Residues 8–15 encoding the "anion hole" of yeast GK are underlined; residues forming hydrogen bonds with GMP are identified with a solid circle; those directly bound are identified with an asterisk.

Table 1. SH3 amino acid homology comparisons

% identical				
PSD-95	dlg	p55	crk	
39 (59)	29 (58)	28 (54)	25 (54)	ZO-1
	59 (81)	37 (66)	42 (65)	PSD-95
		42 (69)	48 (75)	dlg
			35 (63)	p55

Percent identity and percent similarity (in parentheses) for each pair were calculated using the BESTFIT algorithm.

Sequence Unique to ZO-1. The C-terminal 944 residues, immediately following those homologous with the dlg family, are unique. The first 77 residues (positions 806–883) define an acidic domain, pI = 3.72. This is followed by a 853-residue domain, which is slightly less acidic, pI 6.07, and very high in proline (14.4%). Individual proline residues are irregularly spaced and found in groups of up to five. There is no obvious internal repeating organization. Proline-rich domains have been identified in several peripheral membrane proteins, but their functional implications remain undefined (24). An 80-aa domain (α) near the N-terminal boundary of the proline-rich domain is variably present as a result of alternative RNA splicing. The α domain is also proline-rich and indistinguishable from the remainder of this domain with respect to pI and amino acid composition. We have shown that the α-containing isoform is found in most epithelial cell junctions and the α-lacking isoform is found in both endothelial cells and the highly specialized epithelial junctions of renal glomeruli and Sertoli cells of the seminiferous tubules (11, 32). In freeze–fracture electron microscopic images, isoform expression correlates with whether the transmembrane proteins of the junctions break away with the inner or outer plasma membrane leaflet. We hypothesize the α domain is involved in a protein interaction that stabilizes junctions, since junctions expressing ZO-1 without the α domain are more plastic in their ability to open and close the intercellular space and move within the plane of the membrane (11). ZO-1 also immunolocalizes to points of cell–cell contact in cultured nonepithelial cells (astrocytes, myelomas, and fibroblasts; ref. 13), although the specific isoform expressed has not been determined. Movement of ZO-1 from the cytoplasm to membrane is an early event occurring concurrently with cell–cell contact (ref. 19 and unpublished data). These observations suggest that ZO-1 may be involved in transducing a signal required for junction assembly.

CONCLUSION

ZO-1 is a member of a family of proteins implicated in signal transduction at the plasma membrane and specialized cell–cell junctions. A role in signal transduction involving small GTP-binding protein-coupled pathways is implied by the presence of SH3 and GK domains. Such a role is directly supported for the dlg product, which regulates cell proliferation. The colinear distribution of domains within these proteins suggests both a common evolutionary origin and that their integrated function requires coexpression of these domains. We suggest the N-terminal domain of ZO-1, which is homologous to dgl, may be involved in signaling that is predicted to occur during regulation of paracellular permeability or structural reorganization of junctions during development, epithelial cell mitosis, or movement of immune cells across epithelia. The acidic and proline-rich domains of ZO-1 are not shared by other members of the family. Based on the correlation of ZO-1 isoforms with tight junctional properties, we propose that the alternatively spliced C-terminal region of ZO-1 confers structural features specific to tight junctions.

Table 2. GK amino acid homology comparisons

% identical				
PSD-95	dlg	p55	GK	
30 (57)	31 (55)	26 (50)	21 (43)	ZO-1
	71 (88)	30 (58)	37 (56)	PSD-95
		32 (57)	35 (57)	dlg
			35 (59)	p55

Percent identity and percent similarity (in parentheses) for each pair were calculated using the BESTFIT algorithm.

Note Added in Proof. The cDNA sequence of mouse ZO-1 (termed the 220-kDa protein) was recently reported (33) and the protein was shown to colocalize with cadherin in nonepithelial cells.

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