Two Independent Domains of hDlg Are Sufficient for Subcellular Targeting: The PDZ1-2 Conformational Unit and an Alternatively Spliced Domain

Robert A. Lue, Eric Brandin, Erick P. Chan, and Daniel Branton

Department of Molecular and Cellular Biology, Harvard University, Cambridge, Massachusetts 02138

Abstract. hDlg, a human homologue of the *Drosophila* Dlg tumor suppressor, contains two binding sites for protein 4.1, one within a domain containing three PSD-95/Dlg/ZO-1 (PDZ) repeats and another within the alternatively spliced I3 domain. Here, we further define the PDZ–protein 4.1 interaction in vitro and show the functional role of both 4.1 binding sites in situ. A single protease-resistant structure formed by the entirety of both PDZ repeats 1 and 2 (PDZ1-2) contains the protein 4.1–binding site. Both this PDZ1-2 site and the I3 domain associate with a 30-kD NH_2-terminal domain of protein 4.1 that is conserved in ezrin/radixin/moesin (ERM) proteins. We show that both protein 4.1 and the ezrin ERM protein interact with the murine form of hDlg in a coprecipitating immune complex. In permeabilized cells and tissues, either the PDZ1-2 domain or the I3 domain alone are sufficient for proper subcellular targeting of exogenous hDlg. In situ, PDZ1-2–mediated targeting involves interactions with both 4.1/ERM proteins and proteins containing the COOH-terminal T/SXV motif. I3-mediated targeting depends exclusively on interactions with 4.1/ERM proteins. Our data elucidates the multivalent nature of membrane-associated guanylate kinase homologue (MAGUK) targeting, thus beginning to define those protein interactions that are critical in MAGUK function.

T HE human homologue of the *Drosophila discs-large (dlg)*[1] tumor suppressor protein (hDlg; Lue et al., 1994), is a member of a superfamily known as membrane-associated guanylate kinase homologues (MAGUKs) (Woods and Bryant, 1993). The amino acid sequence of hDlg includes a core arrangement of several domains conserved in other MAGUK proteins: a domain that is homologous to the known guanylate kinases (GUK), a Src Homology domain 3 (SH3) motif, and three repetitive sequence motifs known as PSD-95/Dlg/ZO-1 (PDZ) repeats. PDZ sequences (Kennedy, 1995) have previously been described as GLGF repeats (Cho et al., 1992) and Dlg Homology region domains (Bryant et al., 1993). The MAGUK superfamily currently consists of three subgroups distinguished by size and variations in their domain organization (Fig. 1). While the PDZ-SH3-GUK core arrangement is well conserved among family members, several proteins contain additional sequences that are probably essential to their differing cellular functions. For example, hDlg contains an NH_2-terminal domain that is not found in other MAGUKs (Lue et al., 1994), and ZO-1 (Itoh et al., 1993; Willot et al., 1993) and ZO-2 (Jesaitis and Goodenough, 1994) both contain unique COOH-terminal domains. More recently, a rat neuronal homologue of hDlg (SAP97; Muller et al., 1995) has also been shown to contain the additional sequence domains characteristic of the human protein. The observation that recessive mutations in the *Drosophila dlg* locus lead to imaginal disc neoplasia and larval death (Woods and Bryant, 1991) implies that MAGUK proteins have a role in growth control. Aberrant cell adhesion in *dlg* mutants also suggests that MAGUKs provide a functional link between growth regulation and cell–cell contact. More recently, the *Caenorhabditis elegans* LIN-2A MAGUK has been shown to have a role in vulval induction upstream of LET-60 Ras (Horvitz and Sulston, 1980; Ferguson and Horvitz, 1985; Hoskins et al., 1996).

In spite of the mutant studies in *Drosophila* and *C. elegans*, little is known about either the biochemical activities or in vivo function of MAGUK proteins. An early hypothesis of MAGUK function focused on the enzymatic activity of the GUK domain found in all of the known family members. This was partly based on the observation that *Drosophila dlg* mutants with deletions in the GUK domain displayed the mutant phenotype (Woods and Bryant,

---

1. *Abbreviations used in this paper:* dlg, *Drosophila* discs-large; ERM, ezrin/radixin/moesin; GST, glutathione-*S*-transferase; GUK, guanylate kinase; MAGUK, membrane-associated guanylate kinase homologue; PDZ, PSD-95/Dlg/ZO-1; PDZ1-2, a single protease-resistant structure formed by the entirety of both PDZ repeats 1 and 2; SH3, Src Homology domain 3; TB, targeting buffer; T/SXV, threonine/serine, X amino acid, valine.
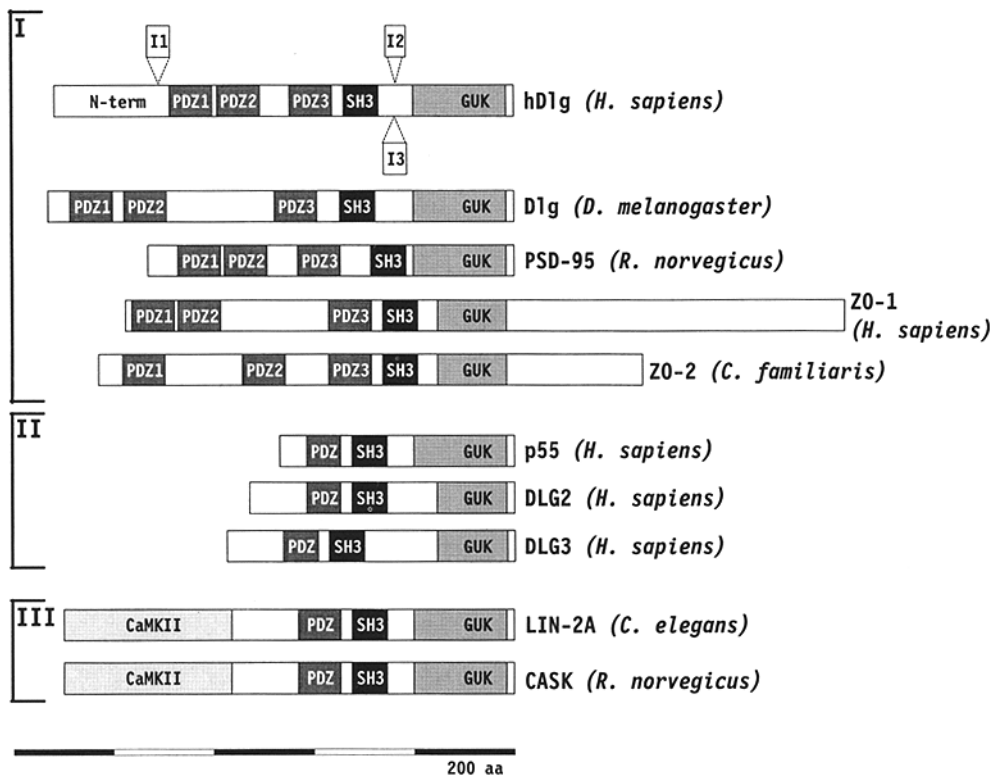
I

| I1 | | | | I2 | | |

N-term | PDZ1 | PDZ2 | PDZ3 | SH3 | GUK | hDlg *(H. sapiens)*

I3

PDZ1 | PDZ2 | PDZ3 | SH3 | GUK | Dlg *(D. melanogaster)*

PDZ1 | PDZ2 | PDZ3 | SH3 | GUK | PSD-95 *(R. norvegicus)*

PDZ1 | PDZ2 | PDZ3 | SH3 | GUK | ZO-1 *(H. sapiens)*

PDZ1 | PDZ2 | PDZ3 | SH3 | GUK | ZO-2 *(C. familiaris)*

II

PDZ | SH3 | GUK | p55 *(H. sapiens)*

PDZ | SH3 | GUK | DLG2 *(H. sapiens)*

PDZ | SH3 | GUK | DLG3 *(H. sapiens)*

III

CaMKII | PDZ | SH3 | GUK | LIN-2A *(C. elegans)*

CaMKII | PDZ | SH3 | GUK | CASK *(R. norvegicus)*

200 aa

Figure 1. Domain organization of the MAGUK superfamily. hDlg, *Drosophila* Dlg (Woods and Bryant, 1991), the rat synaptic protein, PSD-95 (Cho et al., 1992; Kistner et al., 1993), the mammalian tight junction proteins, ZO-1 (Itoh et al., 1993; Willot et al., 1993), and ZO-2 (Jesaitis and Goodenough, 1994), comprise the Group I MAGUK proteins. Dlg and PSD-95 consist entirely of the core arrangement of three PDZ repeats, an SH3 motif, and the GUK domain. hDlg has an additional NH_2-terminal domain that is not found in other MAGUK proteins, and ZO-1 and ZO-2 each include an additional COOH-terminal domain. A rat neuronal form of hDlg termed SAP97 (Muller et al. 1995) and human ZO-2 (Montermini et al., 1995) have also been identified. The human erythrocyte protein, p55 (Ruff et al., 1991), and the human DLG2 and DLG3 gene products (Mazoyer et al., 1995; these sequence data are available from GenBank/EMBL/NDBJ under accession number U37707.) comprise the group II MAGUK proteins. Group II proteins have the same arrangement of conserved domains found in group I but contain only a single incomplete PDZ repeat. Group III MAGUK proteins include the *C. elegans lin-2* gene product (Hoskins et al., 1996) and the rat neuronal CASK protein (Hata et al., 1996). Members of this subgroup are most similar to group II proteins but contain an additional NH_2-terminal domain homologous to the calmodulin-dependent protein kinases (*CaMKII*). The PDZ repeats, SH3, and guanylate kinase domains are labeled. The three alternatively spliced sequence domains found in hDlg are indicated at the approximate positions of their insertion into the known cDNA sequence. The length of each cartoon is proportional to each protein's size.

1991) and the logic of an enzymatic domain having a role in signal transduction. But guanylate kinase enzymatic activity has yet to be demonstrated for any MAGUK protein, and point mutagenesis of the LIN-2A GUK domain does not affect vulval development (Hoskins et al., 1996). Therefore, more serious consideration must be given to the possibility that MAGUKs have an important structural role in the cytoskeleton and that their location and binding interactions are central to their function.

All MAGUK proteins localize to specific cytoplasmic domains of the plasma membrane. hDlg localizes to the inner face of the membrane at regions of intercellular contact (Lue et al., 1994), while Dlg, ZO-1, and ZO-2 are located at occluding cell junctions (Stevenson et al., 1986; Gumbiner et al., 1991; Woods and Bryant, 1991; Jesaitis and Goodenough, 1994). To understand how the subcellular localization of MAGUK proteins can play a role in their function, one must identify the molecular basis of such targeting. The demonstration that both p55 and hDlg bind to cytoskeletal protein 4.1 in vitro offered the first explanation of how MAGUK proteins could directly associate with specific submembrane protein complexes (Lue et al., 1994; Marfatia et al., 1994). Here we show that a single protease-resistant structure formed by both PDZ repeats 1 and 2 (PDZ1-2) forms a single folded conformational unit that constitutes a protein 4.1–binding site. A

second protein 4.1–binding site lies within the I3 domain and is restricted to a subset of alternatively spliced hDlg isoforms. Since both sites on hDlg bind within the conserved 30-kD NH_2-terminal domain of protein 4.1, the association between hDlg and the cytoskeleton should include interactions with other 4.1-related proteins. We show that hDlg is part of a complex that includes direct interactions with both protein 4.1 and the ezrin ERM protein. Either PDZ1-2 or I3 is sufficient for proper submembrane targeting in cells. The targeting of PDZ1-2 depends on both threonine/serine, X amino acid, valine (T/SXV) and 4.1/ERM-mediated protein interactions, while that of the I3 domain depends only on 4.1/ERM protein binding.

Materials and Methods

hDlg Expression Constructs

Plasmid constructs were made that expressed various truncations of the hDlg cDNA as glutathione-*S*-transferase (GST) fusion proteins (Fig. 2). The appropriate sequences were amplified by polymerase chain reaction from human B lymphocyte sscDNA with oligonucleotide primers containing specific restriction enzyme sites. The amplified products were purified, digested with restriction enzymes, and ligated into pGEX-S (Pharmacia LKB Biotechnology, Piscataway, NJ) in-frame for expression as GST fusion proteins in *Escherichia coli*. All constructs were sequenced for accu-
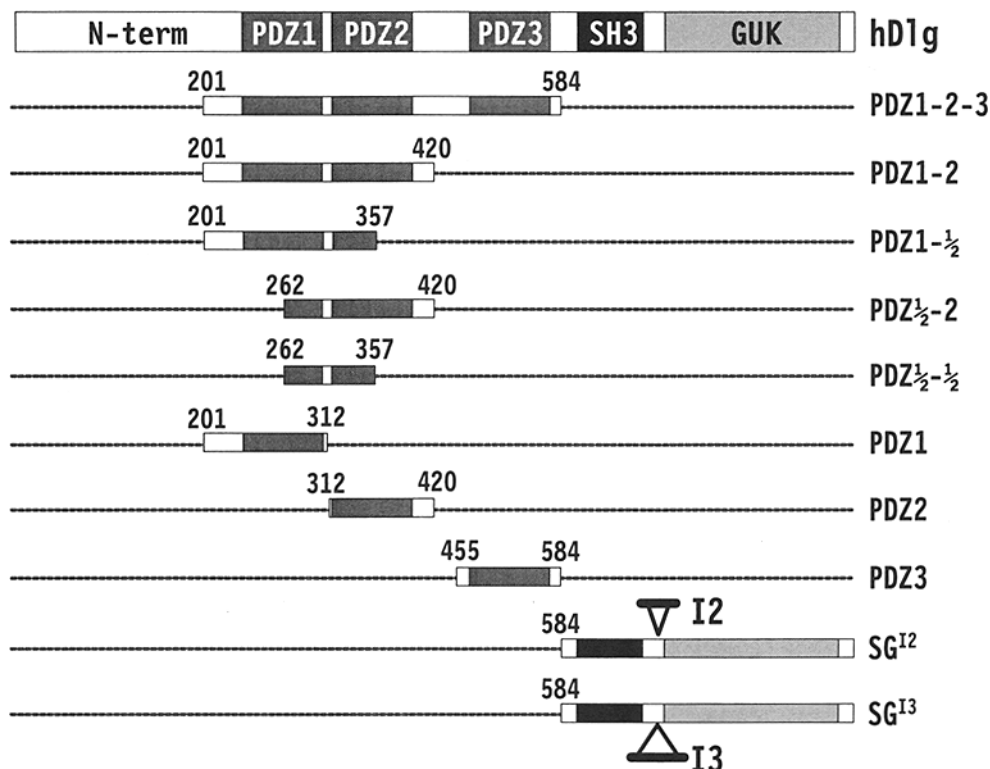
Figure 2. Map of GST-hDlg fusion proteins. Various portions of the hDlg cDNA were subcloned into pGEX-S for expression as NH$_2$-terminal fusions with GST. The amino acid boundaries of each hDlg moiety and the presence of alternatively spliced insertions are indicated for each construct. All constructs included GST at their NH$_2$-termini (not shown).

racy and produced soluble fusion proteins of the expected sizes. The constructs were also characterized to determine which alternatively spliced insertions they contained: SH3/guanylate kinase constructs with either the I2 (GST-SG^{I2}) or I3 (GST-SG^{I3}) domains. The GST-hDlg fusion proteins were expressed and purified according to a protocol modified from Smith and Johnson (1988) and described elsewhere (Lue et al., 1994).

Antibodies

Rabbit polyclonal antibodies 405 and 411 were raised against bacterially expressed and purified portions of hDlg from residues 200–960 and 1–200, respectively. Rabbit polyclonal antibody 14 raised against human erythrocyte protein 4.1 has been described previously (Lue et al., 1994). Both antibodies 405 and 14 were affinity purified by absorption to nitrocellulose immobilized antigen as described (Byers et al., 1987). Rabbit polyclonal antibodies 220, 464, 457, and 454 raised against peptides derived from ezrin, radixin, and moesin are described and numbered in Winckler et al. (1994).

Binding Assays with Protein 4.1

N8-4.1(1), an SP6 eukaryotic expression vector containing a cDNA encoding human B-lymphocyte protein 4.1 (Lue et al., 1994), was expressed in a rabbit reticulocyte lysate supplemented with [^{14}C]leucine according to the manufacturer's directions (Promega Corp., Madison, WI). The size of the radiolabeled protein 4.1 was verified by SDS-PAGE followed by autoradiography. The ^{14}C-labeled protein 4.1 was also purified from the reticulocyte lysate by immunoprecipitation and digested to completion with an excess of α-chymotrypsin. The labeled digestion products were resolved by tricine SDS-PAGE with a continuous gradient from 10–16% polyacrylamide followed by autoradiography. The sizes of the digestion products (30, 16, and 10 kD) matched those predicted for the native protein (Leto and Marchesi, 1984). This implied that the expressed protein 4.1 was properly folded and suitable for use in the binding assays.

The fusion proteins (Fig. 2) were produced and purified as described (Lue et al., 1994) and subsequently immobilized on glutathione agarose beads in binding buffer containing 120 mM KCl, 5 mM sodium phosphate, pH 7.6, 1.0 mM β-mercaptoethanol, and 0.5 mM EDTA. The ratio of fusion protein to beads was normalized to 0.2 μg/μl packed beads for all of the hDlg constructs. 5 μl of reticulocyte lysate containing ^{14}C-labeled 4.1 was added to 30 μl packed beads and incubated at 4°C for 90 min with

gentle mixing. Each binding assay included 10–20 mg/ml of nonspecific blocking proteins from the reticulocyte lysate. The beads were then extensively washed with binding buffer and aspirated dry. Bound ^{14}C-labeled 4.1 was released in hot SDS sample buffer and resolved by SDS-PAGE for autoradiography. Control assays were performed with double the amount of GST alone immobilized on beads. To test for specificity, competitive assays were also performed in the presence of a 10-fold molar excess of the unlabeled 30-kD 4.1 domain purified from human erythrocytes as described (Lue et al., 1994).

Structural Mapping of the PDZ1-2 Domain

Full-length and various truncations of the PDZ1-2 fusion protein were cleaved away from GST as described (Lue et al., 1994). After anion exchange fast protein liquid chromatography purification, the PDZ moieties were dialyzed into digestion buffer containing 20 mM Tris-HCl, pH 7.5, and 50 mM NaCl for α-chymotrypsin, or 20 mM Tris-HCl, pH 8.0, 13 mM NaCl, 1 mM DTT, and 1 mM CaCl$_2$ for trypsin. The proteins at 0.5 mg/ml were then digested at room temperature with α-chymotrypsin or trypsin (Sigma Chemical Co., St. Louis, MO) at an enzyme to substrate molar ratio of 1:200 or 1:100, respectively. Samples were taken at several time intervals between $t = 0$ and $t = 120$ min and immediately boiled in SDS–sample buffer. Equal loadings of the digestion products normalized by protein concentration were resolved by SDS-PAGE.

The SDS–polyacrylamide gels containing the resolved digestion products were electroblotted onto poly(vinylidene difluoride) membranes (BioRad Labs, Hercules, CA). The protease digestion products were then excised from the blot and sequenced on protein sequencer (model 470A; Applied Biosystems Inc., Foster City, CA) with an on-line phenylthiohydantoin analyzer (model 120A; Applied Biosystems, Inc.). The NH$_2$-terminal 10 amino acids were identified for each digestion product. The molecular weights of selected digestion products were determined by electrospray ionization mass spectrometry performed on a triple quadrupole mass spectrometer (Finnigan TSQ700; Microchemistry Facility, Harvard University, Cambridge, MA).

Coimmunoprecipitation

^{35}S-labeled extracts from murine NIH-3T3 cells were prepared from ~5 × 10^7 plated cells that were washed three times in 37°C PBS and then precultured for 4 h in 25 ml DME lacking Met and Cys, supplemented with 10%
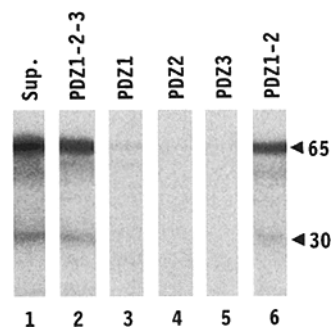
Figure 3. Mapping of the protein 4.1–binding site within the PDZ domain of hDlg. ^{14}C-labeled protein 4.1 was incubated with various GST-hDlg fusion proteins immobilized on beads. The beads were sedimented and washed as described in Materials and Methods. Autoradiograms of the SDS-PAGE resolved ^{14}C-labeled protein 4.1 in the supernatant (lane *1*) and bead pellet (lanes *2-5*) are shown. The immobilized hDlg fusion protein used in each case is indicated above each lane. The positions of the intact protein 4.1 polypeptide (65 kD) and its 30-kD breakdown product are indicated by arrowheads.

dialyzed FBS and 1 mM L-glutamine. 2.5 mCi of ^{35}S-labeled Met and Cys was added to the medium and the incubation continued for another 4 h. The metabolically labeled cells were washed three times in cold TBS and lysed in TBS containing 1% NP-40, 10% glycerol, 2 mM EDTA, 2 μg/ml leupeptin, 1 mM Pefabloc SC, 0.5 μM aprotinin, and 1 μM pepstatin. The dishes were rocked gently on ice for 30 min, and the remaining cells were detached with a rubber policeman. The crude lysate was clarified at 16,000 g at 4°C for 30 min and the supernatant was either used immediately or flash frozen in ethanol/dry ice and stored at −70°C. Lysates were extensively precleared with preimmune rabbit serum followed by protein A–Sepharose before use. In competition assays, the precleared cell lysate was supplemented with purified PDZ1-2 and SG^{13} polypeptides at a final concentration of 250 μg/ml. For each assay, 100 μl of precleared lysate was incubated with 10 μl anti-hDlg affinity purified antibody (405 or 411) on ice for 2 h. Antibody 411 was used in the competition assays because it immunoprecipitates only full-length endogenous hDlg and not the PDZ1-2 and SG^{13} polypeptides. The immune complexes were collected on Sepharose beads conjugated to 50 μg protein A and washed four times with ice cold lysis buffer. The radioactive proteins were resolved by SDS-PAGE and blotted to nitrocellulose. The blots were then either exposed to film or probed with various antibodies. Complexes isolated from 3T3 cells were probed with polyclonal antibodies against human protein 4.1 (14), an epitope shared by ezrin, radixin, and moesin (220), and epitopes unique to either ezrin (464), radixin (457), or moesin (454).

Subcellular Targeting of hDlg Fusion Proteins

Our method was partly based on techniques used to study protein transport into the nucleus and Golgi complex (Beckers et al., 1987; Goda and Pfeffer, 1989; Adam et al., 1990; Moore and Blobel, 1992). Cryosections of normal human colon epithelia were prepared on glass slides. Unfixed human tissues were kindly provided by Youbin Wang and Lan Bo Chen (Harvard Medical School, Boston, MA). Small pieces of tissue were rapidly frozen in isopentane and stored in liquid nitrogen before cryosectioning. 5–10 μm sections were cut in a cryostat, captured on glass slides, and air dried. Frozen tissue sections were thawed at room temperature in PBS before use. CX-1 cells were grown on glass coverslips and washed twice with PBS before use. Cells from both types of preparation were subsequently washed twice in targeting buffer (TB) containing 40 mM Hepes acetate, pH 7.5, 5 mM magnesium acetate, 150 mM potassium acetate, 1 mM EGTA, 10% sucrose, 1% newborn calf serum NCS, 2 mM Pefabloc, 4 μM leupeptin, and 2 μM pepstatin. The cells were then permeabilized in TB with a final concentration of 75 μM digitonin for 30–60 s at room temperature. The permeabilized cells were washed twice in fresh TB and then overlaid with TB supplemented with GST–hDlg fusion protein at a final concentration of 10–50 μg/ml. In competition experiments, the GST–hDlg fusion proteins were preincubated for 2 h before addition to the cells with a 10-fold molar excess of either the native 30-kD protein 4.1 domain (250 μg/ml) or an 11–amino acid peptide (CSNAKAVETDV) derived from the COOH-terminus of the Shaker-type K$^+$ channel (12 μg/ml). After a 1 h incubation with fusion protein at room temperature, the cells were washed three times with fresh TB and excess liquid removed from the coverslip. The cells were then fixed in 100% methanol at −20°C for at least 10 min.

Fixed cells were rinsed twice in PBS, then overlaid with PBS containing a 1:200 dilution of affinity purified rabbit anti-GST polyclonal antibody (385), 1% Tween-20, and 1% BSA, and incubated for 1 h at 37°C. The rabbit anti-protein 4.1 polyclonal antibody (14) and a mouse anti-GST mAb (Santa Cruz Biotechnologies, Santa Cruz, CA) were substituted for 385 in all double-label experiments. The cells were then washed three times in PBS/Tween and overlaid with PBS/Tween containing a 1:250–400 dilution of either FITC-conjugated goat anti–rabbit IgG (Zymed Labs, Inc., S. San Francisco, CA), FITC-conjugated goat anti–mouse IgG, or rhodamine-conjugated goat anti–rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) and 1% BSA and incubated at 37°C for 30 min. The cells were then washed twice in PBS and twice in distilled water. Excess liquid was removed from the coverslip or slide before mounting in SlowFade glycerol medium (Molecular Probes, Eugene, OR). FITC and rhodamine fluorescence patterns were visualized in optical sections taken with a confocal microscope (model M600; BioRad Labs). Four independent preparations of cells or tissue were examined for each applied fusion protein. 250 cells total were counted from five microscope fields per preparation, and the number of cells with detectable immunofluorescence
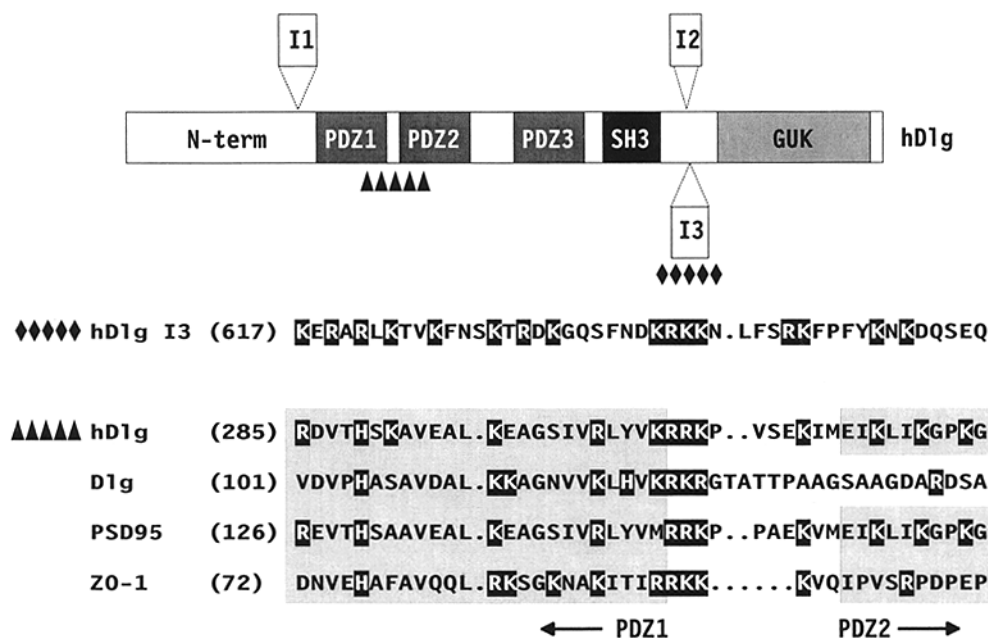

♦♦♦♦♦ hDlg I3 (617) KERARLKTVKFNSKTRDKGQSFNDKRKKN.LFSRKFPFYKNKDQSEQ

▲▲▲▲▲ hDlg (285) RDVTHSKAVEAL.KEAGSIVRLYVKRRKP..VSEKIMEIKLIKGPKG

Dlg (101) VDVPHASAVDAL.KKAGNVVKLHVKRKRGTATTPAAGSAAGDARDSA

PSD95 (126) REVTHSAAVEAL.KEAGSIVRLYVMRRKP..PAEKVMEIKLIKGPKG

ZO-1 (72) DNVEHAFAVQQL.RKSGKNAKITIRRKK......KVQIPVSRPDPEP

◄──── PDZ1   PDZ2 ────►

Figure 4. The PDZ1-2 interrepeat sequence is conserved in a subset of MAGUK proteins. Several group I MAGUK proteins (Fig. 1) contain the closely juxtaposed pair of PDZ repeats with a pattern of positively charged residues between the two repeats (highlighted in black). This pattern is also found in the I3 domain of hDlg.
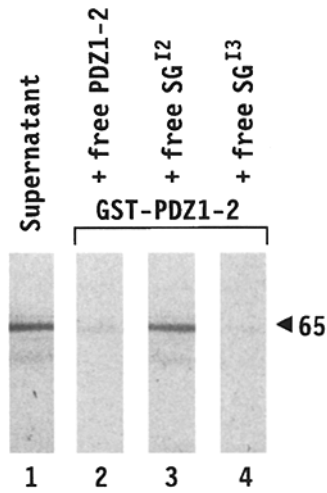
Figure 5. Either free PDZ1-2 or free I3 domain compete with bead bound PDZ1-2C for binding to protein 4.1 ^{14}C-labeled protein 4.1 was incubated with GST-PDZ1-2 fusion protein on beads in the presence of a 10-fold molar excess of the indicated free polypeptides. The beads were sedimented and washed as described in Materials and Methods. Autoradiograms of the SDS-PAGE resolved ^{14}C-labeled protein 4.1 in the supernatant (lane *1*) and bead pellets (lanes *2–4*) are shown. Molecular masses in kD are indicated on the right.

at intercellular junctions was noted. Thus, a total of 1,000 cells were counted for each experimental condition.

Results

PDZ1-2 Binds to Protein 4.1

To determine if a site for protein 4.1 binding was localized to a specific PDZ repeat, various GST–PDZ fusion proteins immobilized on glutathione agarose beads were incubated with ^{14}C-labeled protein 4.1 and then sedimented. The decision to use reticulocyte-expressed protein 4.1 in our binding assays was based on experimental comparisons with *E. coli* expressed protein and the native protein purified from human erythrocytes. Bacterially expressed protein 4.1 was insoluble and thus unsuitable for use in binding assays. Native protein 4.1, which we have used in previous work (Lue et al., 1994), was prone to nonspecific aggregation 1–2 wk after purification. In contrast, the reticulocyte-expressed protein 4.1 could be stored in the lysate at −20°C for up to 9 mo without signs of aggregation or loss of activity. Proper folding of this protein was verified by α-chymotrypsin digestion (see Materials and Methods), and the NH$_2$-terminal 30-kD domain displayed the protease resistance of a well-folded structure. The

specificity of any observed binding using the expressed protein was confirmed by competition with the native human erythroid 30-kD protein 4.1 domain (data not shown).

Our results map protein 4.1-binding to the hDlg domain comprised of PDZ repeats 1 and 2 (Fig. 3). No binding activity was detected with any of the PDZ repeats in isolation. Since both the PDZ1-2 domain and the I3 domains have protein 4.1-binding activity, we compared their amino acid sequences and found a distinctive pattern of positively charged residues in the region between the PDZ1 and PDZ2 repeats that was also present in the I3 domain (Fig. 4). As expected from the similar pattern of charged residues in the PDZ1-2 and I3 domains, both appeared to bind to the same site on protein 4.1. A 10-fold molar excess of free SG^{13} polypeptide (Fig. 2) competed with the PDZ1-2 domain for binding to protein 4.1 (Fig. 5). The site on protein 4.1 to which hDlg binds was previously mapped to the 30-kD NH$_2$-terminal domain (Lue et al., 1994).

PDZ1-2 Forms a Single Conformational and Functional Unit

Our observations suggested that the binding site for protein 4.1 within the PDZ1-2 domain encompassed the boundary between the two repeats. Since the end of PDZ1 and the beginning of PDZ2 defined by sequence alignment were separated by only eight residues, we hypothesized that the first two repeats in fact formed a single functional domain with a folded structure that spanned both. This also suggested that the PDZ repeat boundaries defined by sequence alignment might not coincide with the boundaries of well-folded structural units. Because there are many potential α-chymotrypsin and trypsin sites distributed within the PDZ1-2 sequence (Fig. 6), we tested the stability of the PDZ1-2 domain in the presence of either protease (Fig. 7 *A*). The results showed that PDZ repeats 1 and 2 together form a single stable structure that included all of the PDZ sequences defined by amino acid alignment.

In fact, our determination of the NH$_2$-terminal amino acid sequence together with the molecular weight of the PDZ1-2 digestion product showed that the only α-chymotrypsin cleavages that occurred lay downstream of the predicted COOH-terminal PDZ2 boundary (Fig. 7 *A*). Several potential α-chymotrypsin sites upstream of the predicted

▼ potential chymotrypsin site
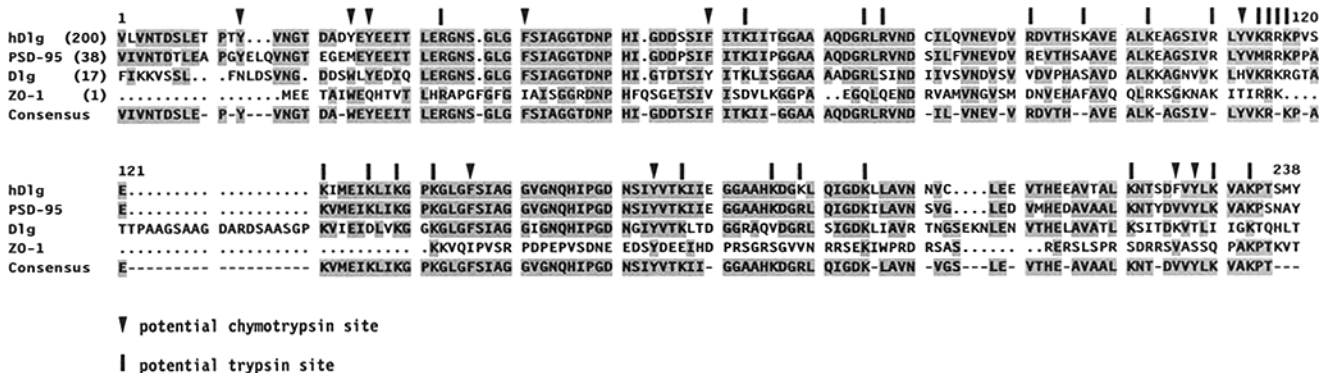
I potential trypsin site

Figure 6. Comparative sequence alignment and protease map of the domain formed by PDZ Repeats 1 and 2. The amino acid sequence of PDZ1-2 from hDlg is aligned with that of PDZ1-2 from *Drosophila* Dlg, rat PSD-95, and human ZO-1. Conserved residues are highlighted in grey, and potential α-chymotrypsin and trypsin sites for hDlg are indicated above its sequence.
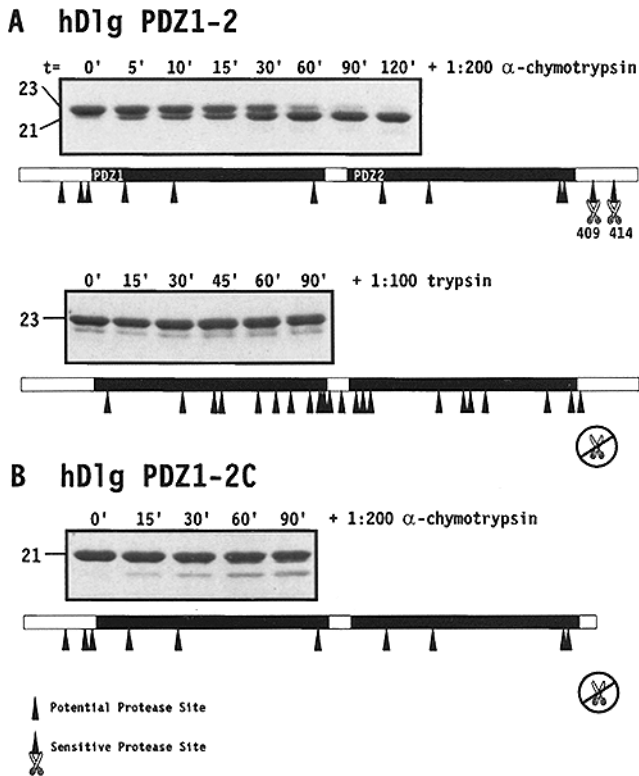
A hDlg PDZ1-2

t= 0' 5' 10' 15' 30' 60' 90' 120' + 1:200 α-chymotrypsin

23
21

0' 15' 30' 45' 60' 90' + 1:100 trypsin

23

B hDlg PDZ1-2C

0' 15' 30' 60' 90' + 1:200 α-chymotrypsin

21

▲ Potential Protease Site

✄ Sensitive Protease Site

Figure 7. PDZ repeats 1 and 2 form a single protease resistant unit (PDZ1-2) that includes all of both repeats. Purified PDZ1-2 peptide was dialyzed into the appropriate enzyme buffer and digested at room temperature with α-chymotrypsin or trypsin at the indicated enzyme to substrate molar ratios. Samples were taken at the time intervals noted and resolved by SDS-PAGE. Digestion products were also gel purified and subjected to NH₂-terminal amino acid sequencing and molecular weight determination by mass spectrometry to define the new boundaries of the digested peptides. Potential protease cleavage sites are diagrammed below each gel. Sites sensitive to digestion are indicated by a pair of scissors and the appropriate residue numbers. Molecular masses in kD are indicated on the left. (*A*) Digestion of PDZ1-2 with α-chymotrypsin and trypsin. (*B*) Digestion of the PDZ1-2C peptide with α-chymotrypsin. The boundaries of PDZ1-2C are those of the PDZ1-2 α-chymotrypsin digestion product.

PDZ1 boundary were resistant to digestion, and the sequence containing these potential sites must also be included in the stable fold of the PDZ1-2 domain. Sequence alignment shows that several of the large MAGUK proteins have conserved many of these residues (Fig. 6). As expected, a PDZ1-2C peptide that had the boundaries derived from the PDZ1-2 digestion product was resistant to α-chymotrypsin digestion (Fig. 7 *B*). After 90 min of digestion at room temperature, >85% of PDZ1-2C was still intact as determined by gel densitometry.

To confirm that the entirety of both repeats was required for the structural integrity of PDZ1-2, we digested peptides that contained all of PDZ1 with an NH₂-terminal portion of PDZ2 (PDZ1-1/2), all of PDZ2 with a COOH-terminal portion of PDZ1 (PDZ 1/2-2), and a COOH-terminal portion of PDZ1 with an NH₂-terminal portion of PDZ2 (PDZ 1/2-1/2). The boundaries of all the digestion products were determined as described above for PDZ1-2.
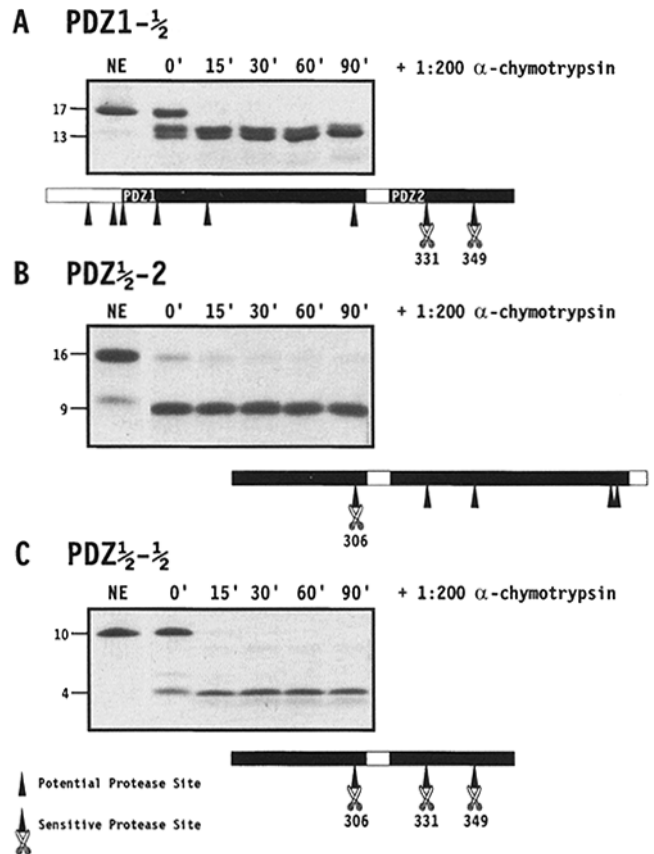
A PDZ1-½

NE 0' 15' 30' 60' 90' + 1:200 α-chymotrypsin

17
13

B PDZ½-2

NE 0' 15' 30' 60' 90' + 1:200 α-chymotrypsin

16
9

C PDZ½-½

NE 0' 15' 30' 60' 90' + 1:200 α-chymotrypsin

10
4

▲ Potential Protease Site

✄ Sensitive Protease Site

Figure 8. The stability of the PDZ1-2 conformational unit requires all of both PDZ repeats. Purified peptides with various truncations of the PDZ1-2C conformational unit were subjected to α-chymotrypsin digestion as described. NH₂-terminal amino acid determination and mass spectrophotometry, when required, were used to define α-chymotrypsin sites sensitive to digestion. Potential protease cleavage sites are diagrammed below each gel. Sites sensitive to digestion are indicated by a pair of scissors and the appropriate residue numbers. Molecular masses in kD are indicated on the left. (*A*) Digestion of PDZ1-1/2 (the complete PDZ1 repeat with the NH₂-terminal half of PDZ2). (*B*) Digestion of PDZ1/2-2 (the complete PDZ2 repeat with the COOH-terminal half of PDZ1). (*C*) Digestion of PDZ1/2-1/2 (the COOH-terminal half of PDZ1 with the NH₂-terminal half of PDZ2).

In the case of PDZ1-1/2 and PDZ 1/2-2, digestion occurred only at potential α-chymotrypsin sites within the incomplete repeat (Fig. 8, *A* and *B*). The single, complete repeats in PDZ1-1/2 and PDZ 1/2-2 were entirely stable. In contrast, PDZ 1/2-1/2 appeared to be unstructured since digestion occurred at all of the potential α-chymotrypsin sites (Fig. 8 *C*). Since PDZ1 and PDZ2 individually folded into stable structures, the stability of PDZ1-2 represented a higher order folding or packing of the two repeats.

To relate the higher order structural fold of PDZ1-2 to its ability to bind protein 4.1, we tested the PDZ1-1/2, PDZ 1/2-2, and PDZ 1/2-1/2 peptides for binding activity. Although all three of the truncated PDZ polypeptides included the complete pattern of interrepeat residues found in PDZ1-2, only full-length PDZ1-2 bound protein 4.1 (Fig. 9). Although the polypeptides used in each assay were shown to be intact by SDS-PAGE (data not shown),
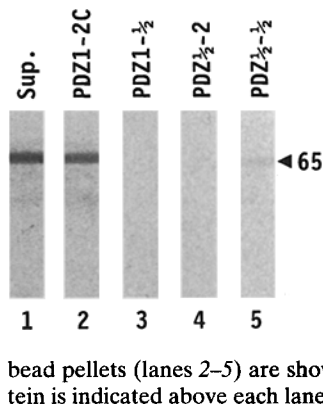
Figure 9. The complete PDZ1-2 conformational unit is required for protein 4.1 binding. ^{14}C-labeled protein 4.1 was incubated with various GST-PDZ fusion proteins on beads. The beads were sedimented and washed as described in Materials and Methods. Autoradiograms of the SDS-PAGE resolved ^{14}C-labeled protein 4.1 in the supernatant (lane *1*) and bead pellets (lanes *2–5*) are shown. The immobilized fusion protein is indicated above each lane.

the mere presence of the interrepeat residues was clearly not sufficient for binding. Rather, the higher order structure of PDZ1-2 was required for protein 4.1–binding activity, and only this structure must present the site in a manner suitable for binding.

hDlg, Protein 4.1, and Ezrin Are Part of a Multiprotein Complex

Since both sites on hDlg bind to the 30-kD NH$_2$-terminal domain of protein 4.1 that is the most conserved motif across a family of proteins (Rees et al., 1990), we tested whether other 4.1/ERM family members might associate with hDlg in cells. A complex consisting of several distinct polypeptides (115, 110, 85, and 75 kD) was immunoprecipitated from murine NIH-3T3 cells by anti-hDlg antibodies (Fig. 10 *A*). Those at 115 and 110 kD were identified as murine homologues of hDlg using two different polyclonal antisera (data not shown). The 85-kD protein was recognized by the anti-protein 4.1 antibody and was the appro-

priate size for an alternatively spliced isoform of protein 4.1 (Conboy et al., 1991). The 85-kD isoform of protein 4.1 also coprecipitated with hDlg from human MCF-7 cell lysates (data not shown). The distinctive 75-kD protein doublet present in the immune complex precipitated from NIH-3T3 cells was recognized by an antibody raised against an epitope shared by all the ERM family members (Fig. 10 *A*, lane *2*), consistent with there being at least two
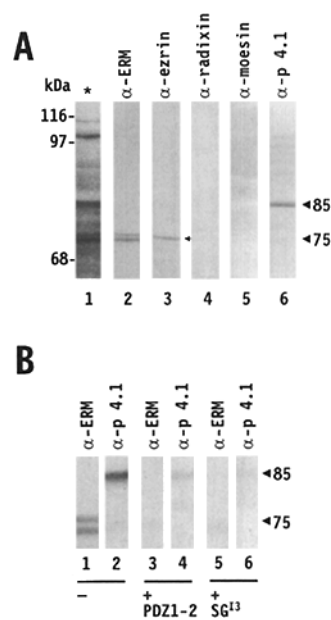

Figure 10. Immunoprecipitation of a protein complex containing hDlg, protein 4.1, and ezrin from murine 3T3 cells. (*A*) ^{35}S-labeled lysates from murine 3T3 cells were immunoprecipitated with anti-hDlg antibody, 405. The isolated immune complex was resolved by SDS-PAGE and blotted to nitrocellulose. An autoradiogram of the blotted proteins is shown in lane *1*. Parallel strips were cut from the same blot and probed with antibodies against an epitope conserved in all ERM proteins, 220 (lane *2*), ezrin, 464 (lane *3*), radixin, 457 (lane *4*), and moesin, 454 (lane *5*). (*B*) Anti-hDlg immunoprecipitations were performed in the presence of the competitor peptides indicated below (see Materials and Methods). Individual lanes from Western blots of the isolated immune complexes were cut in half and each portion was probed with either anti-ERM antibody 220 or anti–protein 4.1 antibody 14 as indicated.
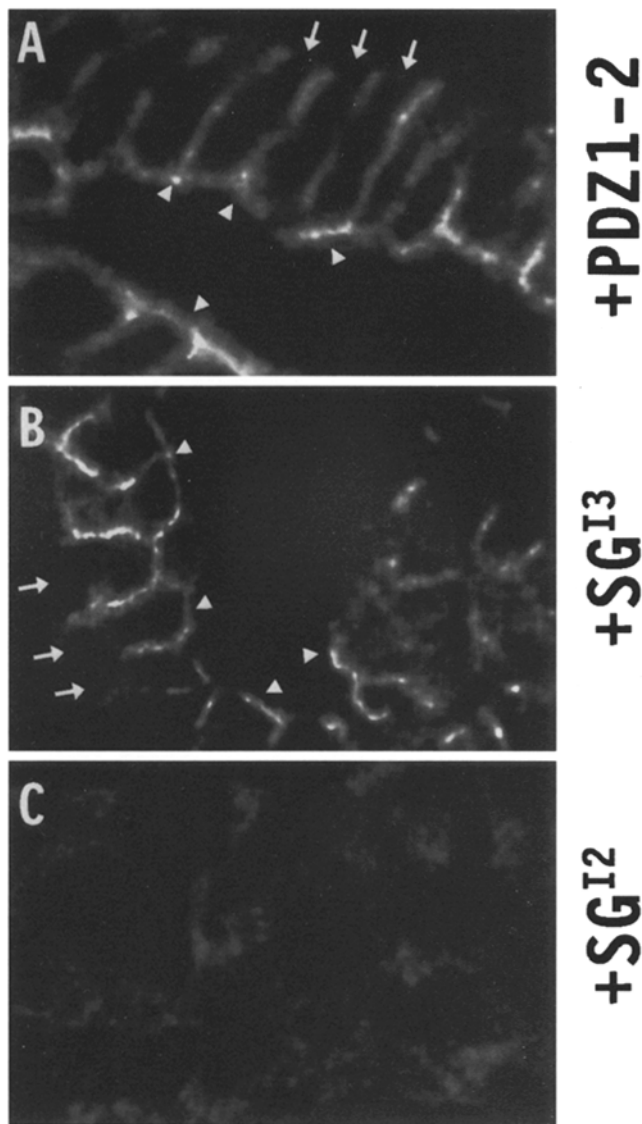

Figure 11. The PDZ1-2 and I3 domains are independently sufficient for proper intracellular targeting. Colon tissue cryosections were permeabilized with digitonin under conditions that preserved cell–cell contact structures. The permeabilized preparations were treated with exogenous GST–hDlg fusion proteins. The localization of bound fusion protein was visualized with anti-GST antibodies followed by FITC-conjugated IgG. Optical sections were taken on a scanning confocal microscope. The applied fusion protein is indicated at the right of each panel. In *A* and *B*, arrow heads indicate basal membrane domains and arrows indicate apical membrane domains.
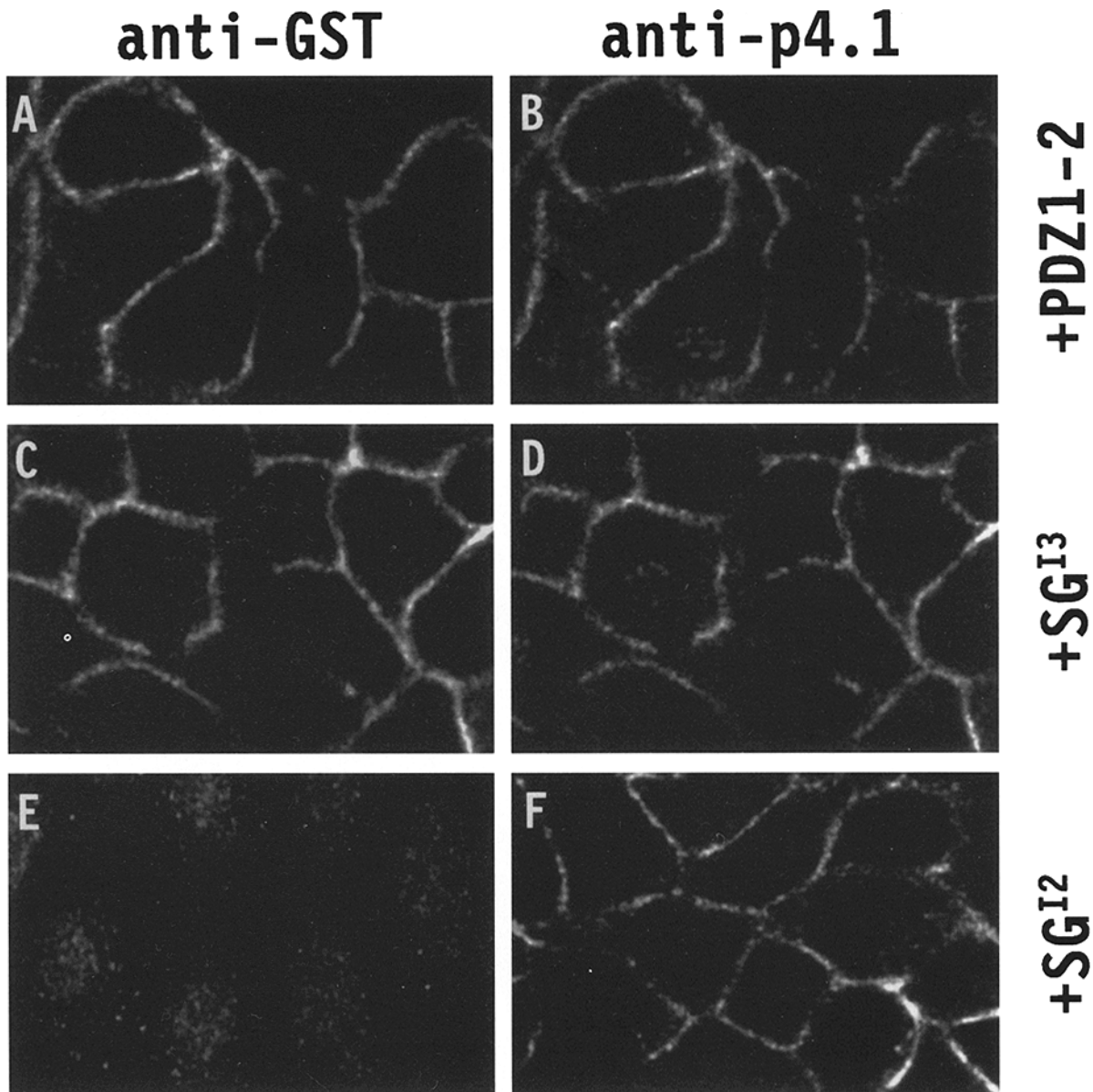
Figure 12. Exogenous PDZ1-2 and I3 domains colocalize with protein 4.1 in CX-1 cells. Human CX-1 cells were permeabilized and treated with exogenous GST-hDlg fusion proteins as described in the Materials and Methods. The localization of the fusion proteins was visualized using a mouse anti-GST mAb followed by FITC-conjugated goat anti–mouse IgG. The colocalization of endogenous protein 4.1 was visualized using a rabbit anti–protein 4.1 polyclonal antibody followed by rhodamine-conjugated goat anti–rabbit IgG. Optical sections at both fluorescence wavelengths were taken on a scanning confocal microscope.

interacting members of the ERM subfamily in the complex. Indeed, most ERM proteins are of similar size and tend to resolve as 75–80-kD bands by SDS-PAGE (Winckler et al., 1994). One of the 75-kD bands was also recognized by an ezrin-specific antibody (Fig. 10 *A*, lane *3*). Specific antibodies against moesin (454) and radixin (457) failed to recognize any of the proteins from the NIH-3T3 precipitates (Fig. 10 *A*, lanes *4* and *5*).

To confirm that both protein 4.1 and the ERM proteins were interacting with the same sites on hDlg, we performed the immunoprecipitation assays in the presence of purified PDZ1-2 and SG^{I3} polypeptides. Since neither of these polypeptides are recognized by the anti-hDlg antibody used in these experiments, PDZ1-2 or SG^{I3} would be expected to compete with endogenous hDlg for binding sites on protein 4.1 and the ERM proteins. Such competition would result in the coprecipitation of reduced amounts of protein 4.1 and the ERM proteins. The PDZ1-2 and SG^{I3} polypeptides individually excluded protein 4.1 and the ERM proteins from the hDlg immune complex (Fig. 10 *B*). These data implied that both ERM proteins, one of which was ezrin, interacted at the same sites on hDlg as protein 4.1. The association of the ERM proteins with hDlg in the immune complex was most likely a direct one because there were no other proteins in the complex at stochiometric levels besides protein 4.1 (Fig. 10 *A*, lane *1*). While the formation of heterodimers between the various ERM proteins has been observed (Gary and Bretscher, 1995), there

| Fusion protein | Localization at intercellular junctions (percentage of total cells) | |
	Cells	Tissue
GST-NT	6	3
GST-PDZ1-2	97	79
GST-PDZ1	3	6
GST-PDZ2	18	16
GST-PDZ3	5	3
GST-SG[12]	4	8
GST-SG[13]	95	88
GST alone	2	1

Four independent cell or tissue preparations were probed with the indicated GST-hDlg fusion proteins. Localized exogenous fusion protein was visualized by immunofluorescence as described. 250 cells total from five microscope fields were counted in each preparation, and the total number of cells per preparation with detectable fluorescent staining at intercellular junctions was determined. Each value represents the average percentage out of a total of 1,000 cells with detectable intercellular junction staining. Standard deviations ranged from 0.5 to 3%.

is no evidence for the direct association of ezrin with protein 4.1.

PDZ1-2 and I3 Are Independently Sufficient for Subcellular Targeting

Given the submembrane colocalization of hDlg and protein 4.1 at sites of intercellular contact (Lue et al., 1994), we tested whether the known protein 4.1–binding domains on hDlg were sufficient for targeting to these sites. Tissue and cultured cells were permeabilized and treated with exogenous GST–hDlg fusion proteins. Successful subcellular targeting of the fusion proteins was ascertained using anti-GST antibodies followed by FITC-conjugated IgG. GST fusion proteins were chosen as probes because of their stability and the signal amplification due to the formation of GST dimers in solution. Several permeabilization conditions with Triton X-100 and digitonin were tested before selecting a procedure that made it possible to introduce exogenous protein without any apparent disruption of cell–cell contact as determined by microscopic observation. Cryosections of human colon epithelia and CX-1 colon carcinoma cells were permeabilized with digitonin in a buffer (see Materials and Methods) that minimized the osmotic disturbance of cellular morphology.

Exogenous GST-PDZ1-2 and GST-SG[13] specifically localized to regions of intercellular contact in colon epithelial tissue and CX-1 cells (Fig. 11, A and B; Fig. 12, A and C). In the case of epithelial tissue, localization was confined to basolateral membrane domains. Exogenous GST, GST-NT, GST-PDZ1, GST-PDZ3, and GST-SG[12] failed to display significant localization to any subcellular structures in either type of preparation. GST-PDZ2 weakly localized to intercellular junctions in 16–18% of the cells counted. In contrast, GST-PDZ1-2 and GST-SG[13] were localized to intercellular junctions in 79 to >95% of the cells counted (Table I). This implied that the PDZ1-2 and I3 domains were independently sufficient for proper targeting to regions of cell–cell contact. Moreover, both domains were also sufficient to account for the specific basolateral targeting in tissue epithelia. hDlg is the only MAGUK protein known to localize at basolateral mem-

brane domains. The failure of GST-SG[12] to correctly localize suggested that the SH3 domain was not involved in the targeting of hDlg to the cytoskeletal structures preserved in our assays. Double-label immunostaining showed that the localization of endogenous protein 4.1 in the permeabilized CX-1 cells was the same as that of the exogenous PDZ1-2 and I3 domains (Fig. 12, B and D). Furthermore, endogenous spectrin immunostaining in permeabilized cells was localized at the plasma membrane, which confirmed that the submembrane cytoskeleton was largely intact (data not shown). No GST immunostaining was detected in unpermeabilized cells treated with exogenous fusion proteins (data not shown).

Competition experiments showed that localization of the PDZ1-2 domain depended on two types of protein–protein interaction. The T/SXV-containing K$^+$ channel peptide, which has been shown to bind PDZ2 in hDlg (Kim et al., 1995), did not significantly compete for PDZ1-2 targeting (Fig. 13 A), while the 30-kD protein 4.1 domain only weakly competed for PDZ1-2 targeting (Fig. 13 C). In contrast, when the competitors were applied together, PDZ1-2 did not target to cell–cell contact sites in CX-1 cells (Fig. 13 E). Therefore, both 4.1/ERM protein and T/SXV-dependent mechanisms played a role in the subcellular targeting of PDZ1-2. On the other hand, the 30-kD domain of protein 4.1 effectively competed for the subcellular targeting of the I3 domain (Fig. 13 D). Therefore, I3 domain targeting depended solely on a 4.1/ERM protein-dependent mechanism.

Discussion

Our data indicate that the targeting of hDlg to the membrane cytoskeleton is dependent on PDZ repeats 1 and 2 and the I3 domain. Although SH3 domains are known to bind polyproline motifs found in many signaling molecules, and the SH3 domain of phospholipase C-γ is essential for targeting to the actin cytoskeleton (Bar-Sagi et al., 1993), the SH3 domain of hDlg is not essential for membrane targeting in our assay system.

All of the MAGUK proteins known contain from one to three ~100–amino acid PDZ repeats. Conserved PDZ repeats have also been found in several proteins that are not members of the MAGUK family, such as syntrophin and the family of protein tyrosine phosphatases typified by PTP-BAS (for review see Ponting and Phillips, 1995). Our demonstration that the PDZ-protein 4.1–binding interaction requires the entirety of both PDZ1 and PDZ2 is consistent with the fact that both repeats together form a single higher-order conformational unit. The observation that PDZ1-2 is able to target to sites of intercellular contact far more efficiently than PDZ2 alone further supports the notion that the higher-order structure of PDZ1-2 has a functional role. Although recent data shows that single PDZ repeats can bind to several membrane receptors (Kim et al., 1995; Kornau et al., 1995; Sato et al., 1995) and to the APC tumor suppressor protein (Matsumine et al., 1996), the K$^+$ channel binds more strongly to the PDZ1-2 sequence than to PDZ2 alone (Kim et al., 1995). While the K$^+$ channel–binding site is fully contained within PDZ2, the higher-order structure of PDZ1-2 may present the site more appropriately for binding to the channel.
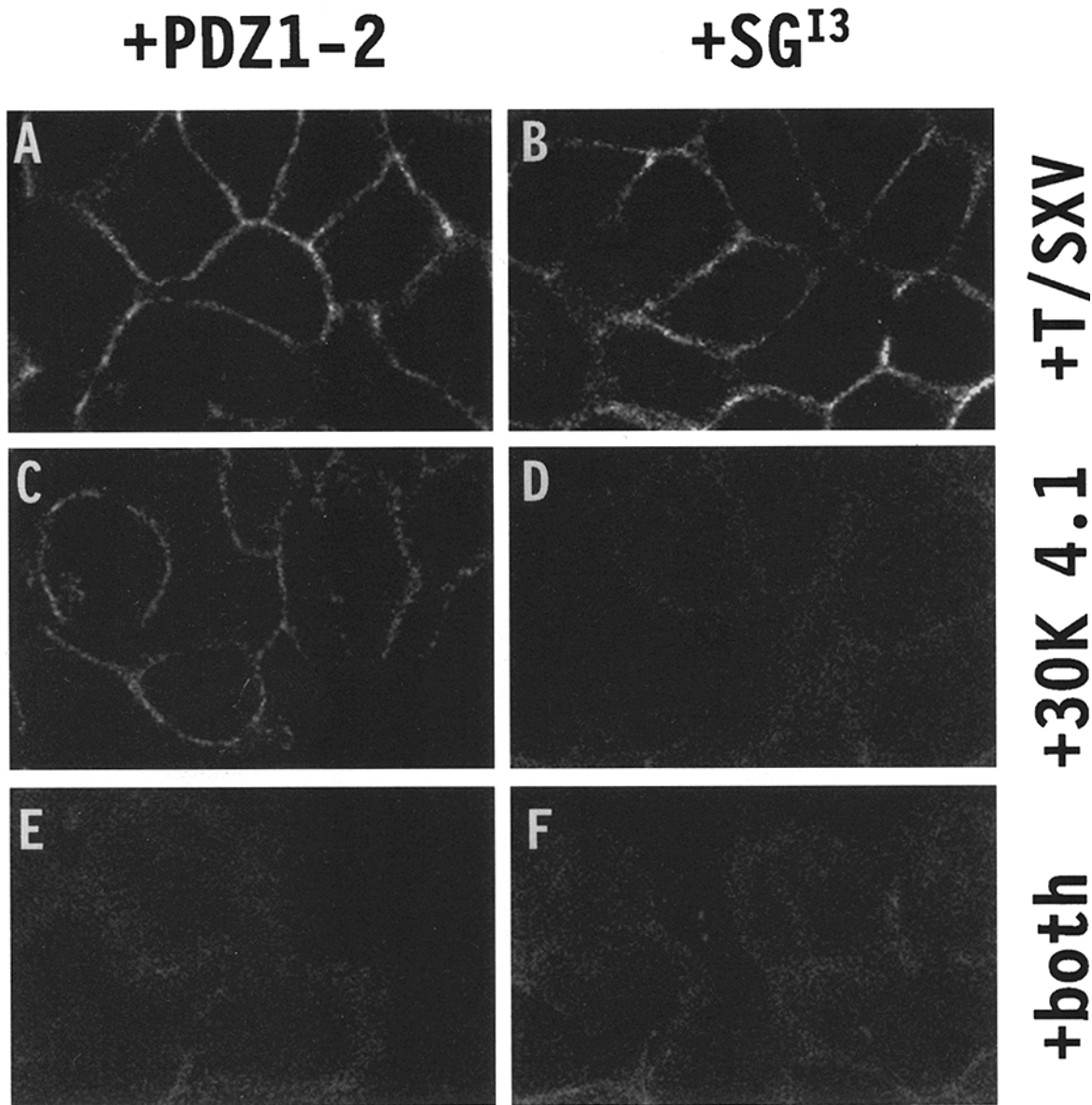
+PDZ1-2   **+SG^{I3}**

Figure 13. Types of protein interactions involved in the intracellular targeting of the PDZ1-2 and I3 domains. Permeabilized CX-1 cells were treated with exogenous GST–hDlg fusion proteins that were preincubated with the indicated competitor peptides (see Materials and Methods). The localization of bound fusion protein was visualized with anti-GST antibodies followed by FITC-conjugated IgG. Optical sections were taken on a scanning confocal microscope.

The conservation of protein 4.1–binding activity among the PDZ1-2 and I3 sequences of hDlg and the site in p55 (Marfatia et al., 1995) may depend on the pattern of basic residues common to all three domains since their amino acid sequences are otherwise not homologous. Homologous patterns of basic residues also occur in *Drosophila* Dlg and human DLG2, between the SH3 and GUK domains of their respective proteins. While it remains to be determined whether the homologous sites in other MAGUK proteins also bind to protein 4.1, conserved 4.1/ERM protein–binding activity is likely to be one mechanism for linking MAGUK proteins to the membrane cytoskeleton.

The structural presentation of the PDZ1-2– and I3 domain–binding sites for protein 4.1 appear to differ substantially. In the case of the PDZ1-2 domain, the protein 4.1–

binding site is part of a well-folded structure that protects the peptide backbone from protease digestion. In contrast, the I3 domain displays the proteolytic sensitivity characteristic of a flexible loop (data not shown). The phenomenon of a conserved protein binding motif being displayed in the context of differing secondary structures is also true of the integrin–binding RGD motif (for review see Haas and Plow, 1994), where differences in the conformational presentation of the RGD motif are thought to result in altered binding affinity or specificity for integrins (Main et al., 1992; Haas and Plow, 1994; Krezel et al., 1994). It will be of interest to determine whether the PDZ1-2 and I3 domains of hDlg display differences in affinity or specificity for members of the 4.1/ERM family that depend on the conformational presentation of the binding site.
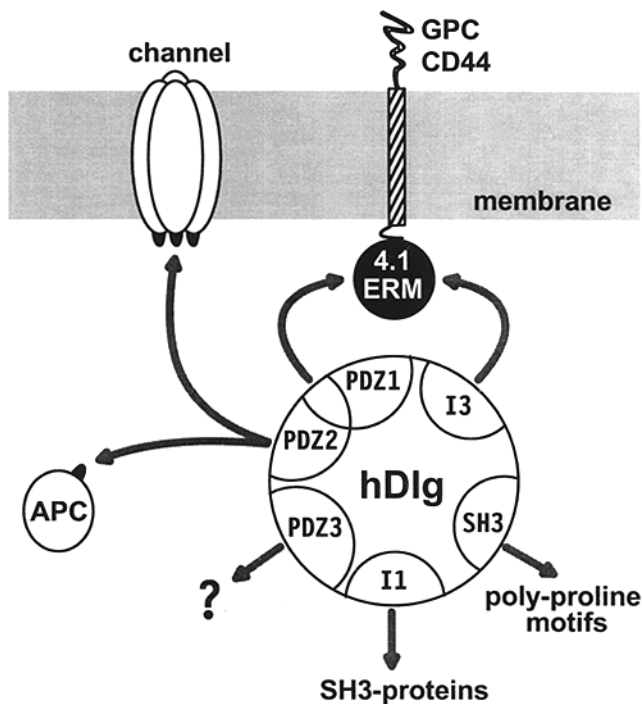
channel

GPC
CD44

membrane

4.1
ERM

PDZ1

I3

PDZ2

hDlg

APC

PDZ3

SH3

?

I1

poly-proline
motifs

SH3-proteins

Figure 14. Model of possible protein–protein associations within an hDlg-dependent complex. Diagramatic representation of an hDlg-dependent protein complex associated with the membrane via interactions with 4.1/ERM family members and membrane glycoproteins. Four potential sites for protein–protein interactions besides the PDZ1-2 and I3 domains are represented. PDZ2 binds the cytoplasmic portion of the Shaker-type K^+ channel (Kim et al., 1995) and is likely to interact with other channel proteins with the T/SXV motif (Kornau et al., 1995). T/SXV-containing cytoplasmic proteins such as the APC tumor suppressor also associate with PDZ2 (Matsumine et al., 1996). PDZ3 is likely to participate in as yet undefined protein–protein interactions that may include homotypic binding to PDZ repeats in other proteins (Brenman et al., 1996). The alternatively spliced I1 domain is predicted to form a poly-proline II helix and binds SH3 motifs commonly found in both cytoskeletal and signaling proteins (Lue, R., unpublished results). The SH3 motif of hDlg probably binds to poly-proline sequences in other signaling molecules. Blot binding assays indicate that it does not bind to the poly-proline sequence of I1 (Lue, R., unpublished results).

The fact that ezrin is one of a limited number of proteins that coprecipitates with hDlg supports the idea that different but related proteins could bind at hDlg's two protein 4.1-binding sites. The 30-kD NH_2-terminal domain of protein 4.1 to which hDlg binds (Lue et al., 1994) is well conserved across a superfamily of related proteins including ezrin (Gould et al., 1989), talin (Rees et al., 1990), the human protein tyrosine phosphatase PTPase MEG (Gu et al., 1991), and the NF2 gene product, merlin/schwannomin (Rouleau et al., 1993; Trofatter et al., 1993). The association of hDlg with members of the 4.1/ERM family could account for its membrane localization since many family members bind to integral membrane proteins. Human erythrocyte protein 4.1 links the spectrin network to the membrane via glycophorin C (Anderson and Lovrien, 1984; Leto et al., 1986). Ezrin, moesin, and radixin all associate with CD44, a widely distributed 140-kD integral

membrane glycoprotein (Sato et al., 1992; Tsukita et al., 1994), which may represent an alternative way by which protein 4.1–related proteins associate with the membrane. We have shown similar localization patterns for human protein 4.1 and hDlg (Lue et al., 1994), and *Drosophila* protein 4.1 has been localized to septate junctions (Fehon et al., 1994) precisely where Dlg is located (Woods and Bryant, 1991). This suggests that the interaction between MAGUK proteins and members of the 4.1/ERM family is functionally relevant in both vertebrates and invertebrates.

Our results show that the subcellular targeting of hDlg depends on several overlapping protein interactions. Submembrane domains such as those at focal adhesions and tight junctions are commonly characterized by large complexes of cytoskeletal and signaling proteins (for review see Yamada and Miyamoto, 1995; Tsukita et al., 1993). Our demonstration that the two 4.1/ERM protein–binding domains of hDlg, PDZ1-2 and I3, are sufficient for subcellular targeting defines one of the important protein–protein interactions involved in MAGUK localization. The association of PDZ1-2 with a T/SXV-containing protein represents another mechanism for localization. It is noteworthy that hDlg colocalizes with the adenomatus polyposis coli (APC) tumor suppressor protein at basolateral submembrane domains via just such a T/SXV-mediated interaction (Matsumine et al., 1996). It will be of interest to identify the single or multiple T/SXV-containing proteins that are involved in the PDZ1-2–dependent targeting of hDlg.

The protein interactions described here could allow for the cross-linking of hDlg molecules and their associated proteins. In one scenario, the bivalent interaction of hDlg with 4.1/ERM family members could form a physical network of hDlg molecules and cross-link associated proteins. Alternatively, the ability of PDZ1-2 to associate with T/SXV motifs while the I3 domain associates with 4.1/ERM proteins would also allow for the cross-linking of hDlg molecules. Thus, hDlg (as well as other MAGUK proteins) may have a role in nucleating protein complexes at defined submembrane domains (Fig. 14). This is consistent with the involvement of PSD-95 in the proper clustering of Shaker-type K^+ channels (Kim et al., 1995). Membrane channels clustered with MAGUK proteins could assemble into specific membrane domains via interactions between MAGUKs and 4.1/ERM family members. Other data implies that PSD-95 could also cluster nitric oxide synthase with the NMDA receptor by homotypic, PDZ–PDZ binding interactions (Brenman et al., 1996).

Both the composition and localization of a MAGUK-dependent protein complex will depend on the complement of binding domains contained in that specific MAGUK. There are substantial variations in the domain complement between members of the different MAGUK subgroups (Fig. 1) and between alternatively spliced isoforms of hDlg. hDlg contains at least two alternatively spliced isoform–dependent protein-binding domains. The I1 domain in the NH_2 terminus binds to SH3 domains from other proteins (Lue, R., unpublished results) and the I3 domain binds to protein 4.1 (Lue et al., 1994). This suggests that various hDlg isoforms can associate with different groups of proteins and consequently have distinct functions. Since PDZ repeats 1 and 2 are structurally and functionally distinct from repeat 3, different types of PDZ-

mediated protein interactions may occur between hDlg and other proteins. Indeed, the fact that various proteins contain differing numbers of PDZ repeats may reflect differences in the relative complexity of their protein interactions. It will be of interest to determine whether various hDlg isoforms also display subtle differences in localization based on the presence of both PDZ1-2 and I3 versus just PDZ1-2 alone. Once targeted to the membrane via binding to 4.1/ERM family members or T/SXV receptors, binding interactions with other proteins at individual PDZ repeats, at the SH3 motif, or at the I1 domain are likely to either regulate or directly enable hDlg function.

References

Adam, S.A., R. Sterne Marr, and L. Gerace. 1990. Nuclear import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111: 807–816.

Anderson, R.A., and E.F. Lovrien. 1984. Glycophorin is linked by band 4.1 protein to the human erythrocyte membrane skeleton. *Nature (Lond.).* 307:655–658.

Bar-Sagi, D., D. Rotin, A. Batzer, V. Mandiyan, and J. Schlessinger. 1993. SH3 domains direct cellular localization of signaling molecules. *Cell.* 74:83–91.

Beckers, C.J.M., D.S. Keller, and W.E. Balch. 1987. Semi-intact cells permeable to macromolecules: use in reconstitution of protein transport from the endoplasmic reticulum to the Golgi complex. *Cell.* 50:523–534.

Brenman, J.E., D.S. Chao, S.H. Gee, A.W. McGee, S.E. Craven, D.R. Santillano, Z. Wu, F. Huang, H. Xia, M.F. Peters, et al. 1996. Interaction of nitric oxide synthase with the postsynaptic density protein PSD-95 and O1-syntrophin mediated by PDZ domains. *Cell.* 84:757–767.

Bryant, P.J., K.L. Watson, R.W. Justice, and D.F. Woods. 1993. Tumor suppressor genes encoding proteins required for cell interactions and signal transduction in Drosophila. *Development (Camb.).* (Suppl.)239–249.

Byers, T.J., R. Dubreuil, D.P. Kiehart, L.S.B. Goldstein, and D. Branton. 1987. *Drosophila* spectrin II. Conserved features of the α-subunit are revealed by analysis of cDNA clones and fusion proteins. *J. Cell Biol.* 105:2103–2110.

Cho, K.-O., C.A. Hunt, and M.B. Kennedy. 1992. The rat brain postsynaptic density fraction contains a homolog of the Drosophila discs-large tumor suppressor protein. *Neuron.* 9:929–942.

Conboy, J.G., J.Y. Chan, J.A. Chasis, Y.W. Kan, and N. Mohandas. 1991. Tissue- and development-specific alternative RNA splicing regulates expression of multiple isoforms of erythroid membrane protein 4.1. *J. Biol. Chem.* 266:8273–8280.

Fehon, R.G., I.A. Dawson, and S. Artavanis-Tsakonas. 1994. A Drosophila homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the coracle gene. *Development (Camb.).* 120:545–557.

Ferguson, E.L., and H.R. Horvitz. 1985. Identification and characterization of 22 genes that affect the vulval cell lineages of the nematode Caenorhabditis elegans. *Genetics.* 110:17–72.

Gary, R., and A. Bretscher. 1995. Ezrin self-association involves binding of an N-terminal domain to a normally masked C-terminal domain that includes the F-actin binding site. *Mol. Biol. Cell.* 6:1061–1075.

Goda, Y., and S. Pfeffer. 1989. Cell-free systems to study vesicular transport along the secretory and endocytic pathways. *FASEB J.* 3:2488–2495.

Gould, K.L., A. Bretscher, F.S. Esch and T. Hunter. 1989. cDNA cloning and sequencing of the protein-tyrosine kinase substrate, ezrin, reveals homology to band 4.1. *EMBO (Eur. Mol. Biol. Organ.) J.* 8:4133–4142.

Gu, M., J.D. York, I. Warshawsky, and P.W. Majerus. 1991. Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. *Proc. Natl. Acad. Sci. USA.* 88:5867–5871.

Gumbiner, B., T. Lowenkopf, and D. Apatira. 1991. Identification of a 160 kD polypeptide that binds to the tight junction protein ZO-1. *Proc. Natl. Acad. Sci. USA.* 88:3460–3464.

Haas, T.A., and E.F. Plow. 1994. Integrin-ligand interactions: a year in review. *Curr. Opin. Cell Biol.* 6:656–662.

Hata, Y., S. Butz, and T.C. Sudhof. 1996. CASK: a novel *dlg*/PSD95 homolog with an N-terminal calmodulin-dependent protein kinase domain identified by interaction with neurexins. *J. Neurosci.* 16:2488–2494.

Horvitz, H.R., and J.E. Sulston. 1980. Isolation and genetic characterization of

cell-lineage mutants of the nematode *Caenorhabditis elegans. Genetics.* 96: 435–454.

Hoskins, R., A.F. Hajnal, S.A. Harp, and S.K. Kim. 1996. The *C. elegans* vulval induction gene *lin-2* encodes a member of the MAGUK family of cell junction proteins. *Development (Camb.).* 122:97–111.

Itoh, M., A. Nagafuchi, S. Yonemura, T. Kitani-Yasuda, S. Tsukita, and S. Tsukita. 1993. The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. *J. Cell Biol.* 121:491–502.

Jesaitis, L.A., and D.A. Goodenough. 1994. Molecular characterization and tissue distribution of ZO-2, a tight junction protein homologous to ZO-1 and the Drosophila discs-large tumor suppressor protein. *J. Cell Biol.* 124:949–961.

Kennedy, M.B. 1995. Origin of PDZ (DHR, GLGF) domains. *TIBS (Trends Biochem. Sci.).* 20:350.

Kim, E., M. Niethammer, A. Rothschild, Y.N. Jan, and M. Sheng. 1995. Clustering of Shaker-type K$^+$ channels by interaction with a family of membrane-associated guanylate kinases. *Nature (Lond.).* 378:85–88.

Kistner, U., B.M. Wenzel, R.W. Veh, C. Cases-Langhoff, A.M. Garner, U. Appeltauer, B. Voss, E.D. Gundelfinger, and C.C. Garner. 1993. SAP90, a rat presynaptic protein related to the product of the Drosophila tumor suppressor gene dlg-A. *J. Biol. Chem.* 268:4580–4583.

Kornau, H-C., L.T. Schenker, M.B. Kennedy, and P.H. Seeburg. 1995. Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95. *Science (Wash. DC).* 269:1737–1740.

Krezel, A.M., G. Wagner, J. Seymour-Ulmer, and R.A. Lazarus. 1994. Structure of the RGD protein decorsin: conserved motif and distinct function in Leech proteins that affect blood clotting. *Science (Wash. DC).* 264:1944–1947.

Leto, T.L., and V.T. Marchesi. 1984. A structural model of human erythrocyte protein 4.1. *J. Biol. Chem.* 259:4603–4608.

Leto, T.L., B.M. Pratt, and J.A. Madri. 1986. Mechanisms of cytoskeletal regulation: modulation of aortic endothelial cell protein band 4.1 by the extracellular matrix. *J. Cell. Physiol.* 127:423–431.

Lue, R.A., S.M. Marfatia, D. Branton, and A.H. Chishti. 1994. Cloning and characterization of hDlg: the human homologue of the Drosophila discs large tumor suppressor binds to protein 4.1. *Proc. Natl. Acad. Sci. USA.* 91: 9818–9822.

Main, A.L., T.S. Harvey., M. Baron, J. Boyd, and I.D. Campbell. 1992. The three-dimensional structure of the tenth type III module of fibronectin: an insight into RGD-mediated interactions. *Cell.* 71:671–678.

Marfatia, S.M., R.A. Lue, D. Branton, and A.H. Chishti. 1994. In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1 and glycophorin C. *J. Biol. Chem.* 269:8631–8634.

Marfatia, S.M., R.A. Lue, D. Branton, and A.H. Chishti. 1995. Identification of the protein 4.1 binding interface on glycophorin C and p55, a homologue of the Drosophila discs-large tumor suppressor protein. *J. Biol. Chem.* 270: 715–719.

Matsumine, A., A. Ogai, T. Senda, N. Okumura, K. Satoh, G-H. Baeg, T. Kawahara, S. Kobayashi, M. Okada, K. Toyoshima, and T. Akiyama. 1996. Binding of APC to the human homolog of the Drosophila Discs Large tumor suppressor protein. *Science (Wash. DC).* 272:1020–1023.

Mazoyer, S., S.A. Gayther, M.A. Nagai, S.A. Smith, A. Dunning, E.J. VanRensburg, H. Albertsen, R. White, and B.A. Ponder. 1995. A gene (DLG2) located at 17q12-q21 encodes a new homologue of the Drosophila tumor suppressor dlg-A. *Genomics.* 28:25–31.

Montermini, L., F. Rodius, L. Pianese, M.D. Molto, M. Cossee, V. Campuzano, F. Cavalcanti, A. Monticelli, F. Palau, G. Gyapay, et al. 1995. The Friedreich ataxia critical region spans a 150-kb interval on chromosome 9q13. *Am. J. Hum. Genet.* 57:1061–1067.

Moore, M.S., and G. Blobel. 1992. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell.* 69:939–950.

Muller, B.M., U. Kistner, R.W. Veh, C. Cases-Langhoff, B. Becker, E.D. Gundelfinger, and C.C. Garner. 1995. Molecular characterization and spatial distribution of SAP97, a novel presynaptic protein homologous to SAP90 and the Drosophila discs-large tumor suppressor protein. *J. Neurosci.* 15:2354–2366.

Ponting, C.P., and C. Phillips. 1995. DHR domains in syntrophins, neuronal NO synthases and other intracellular proteins. *TIBS (Trends Biochem. Sci.).* 20: 102–103.

Rees, D.J.G., S.E. Ades, S.J. Singer, and R.O. Hynes. 1990. Sequence and domain structure of talin. *Nature (Lond.).* 347:685–689.

Rouleau, G.A., P. Merel, M. Lutchman, M. Sanson, J. Zucman, C. Marineau, K. Hoang-Xuan, S. Demczuk, C. Desmaze, B. Plougastel, et al. 1993. Alteration in a new gene encoding a putative membrane-organizing protein causes neurofibromatosis type 2. *Nature (Lond.).* 363:515–521.

Ruff, P., D.W. Speicher, and A. Husain-Chishti. 1991. Molecular identification of a major palmitoylated erythrocyte membrane protein containing the src homology 3 motif. *Proc. Natl. Acad. Sci. USA.* 88:6595–6599.

Sato, N., N. Funayama, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1992. A gene family consisting of ezrin, radixin and moesin. Its specific localization at actin filament/plasma membrane association sites. *J. Cell Sci.* 103: 131–143.

Sato, T., S. Irie, S. Kitada, and J.C. Reed. 1995. FAP-1: a protein tyrosine phosphatase that associates with Fas. *Science (Wash. DC).* 268:411–415.

Smith, D.B., and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. *Gene.* 67:31–40.

Stevenson, B.R., J.D. Siliciano, M.S. Mooseker, and D.A. Goodenough. 1986. Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.

Trofatter, J.A., M.M. MacCollin, J.L. Rutter, J.R. Murrell, M.P. Duyao, D.M. Parry, R. Eldridge, N. Kley, A.G. Menon, K. Pulaski, et al. 1993. A novel moesin-, ezrin-, radixin-like gene is a candidate for the neurofibromatosis 2 tumor suppressor. *Cell.* 72:791–800.

Tsukita, S., M. Itoh, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1993. Submembranous junctional plaque proteins include potential tumor suppressor molecules. *J. Cell Biol.* 123:1049–1053.

Tsukita, S., K. Oishi, N. Sato, J. Sagara, A. Kawai, and S. Tsukita. 1994. ERM family members as molecular linkers between the cell surface glycoprotein CD44 and actin-based cytoskeletons. *J. Cell Biol.* 126:391–401.

Willott, E., M.S. Balda, A.S. Fanning, B. Jameson, C. Van Itallie, and J.M. Anderson. 1993. The tight junction protein ZO-1 is homologous to the Drosophila discs-large tumor suppressor protein of septate junctions. *Proc. Natl. Acad. Sci. USA.* 90:7834–7838.

Winckler, B., Ch. Gonzalez Agosti, M. Magendantz, and F. Solomon. 1994. Analysis of a cortical cytoskeletal structure: a role for ezrin-radixin-moesin (ERM proteins) in the marginal band of chicken erythrocytes. *J. Cell Sci.* 107:2523–2534.

Woods, D.F., and P.J. Bryant. 1991. The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. *Cell.* 66:451–464.

Woods, D.F., and P.J. Bryant. 1993. ZO-1, DlgA and PSD-95/SAP90: homologous proteins in tight, septate and synaptic cell junctions. *Mech. Dev.* 44:85–89.

Yamada, K.M., and S. Miyamoto. 1995. Integrin transmembrane signaling and cytoskeletal control. *Curr. Opin. Cell Biol.* 7:681–689.