The Distribution and Function of Alternatively Spliced Insertions in hDlg*

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hDlg is the human homolog of the Drosophila Disc-large tumor suppressor. As a member of the MAGUK (membrane-associated guanylate kinase) family of scaffolding proteins, hDlg is composed of three PDZ (PSD-95, Dlg, and ZO-1) repeats, an SH3 (Src homology 3) motif, and a GUK (guanylate kinase-like) domain. Additionally, hDlg contains two regions of alternative splicing. Here we identify a novel insertion, I1B, located N-terminal to the PDZ repeats. We further analyze the tissue-specific combinations of insertions and correlate those results with the distribution of protein isoforms. We also identify the functions of the two alternatively spliced regions. The N-terminal alternatively spliced region is capable of binding several SH3 domains and also moderates the level of protein oligomerization. Insertions in the second region are responsible for determining the localization of hDlg, with insertion I3 targeting the protein to the membrane regions of cell-cell contact and insertion I2 targeting the protein to the nucleus.

First identified from human B lymphocyte cDNA, hDlg encodes a 100-kDa protein sharing 60% similarity with Drosophila Dlg and 70% similarity with rat SAP90/PSD-95 (1). Both hDlg mRNA and protein are abundant in human and murine tissues (1). Consistent with the cellular localization of Dlg to septate junctions, hDlg localizes to regions of cell-cell contact in human epithelial cells (1). Dlg and hDlg are members of the MAGUK1 family of proteins typically involved in protein scaffolding and cell signaling. MAGUK family members contain three core domains: either one or three PDZ repeats, an SH3 motif, and a GUK domain homologous to guanylate kinase enzymes.

hDlg has been shown to interact with a number of cytosolic structural proteins. Protein 4.1 also bind to hDlg and, in turn, form a complex with the membrane glycoprotein CD44 (2). The GUK domain recruits a number of proteins including the GKAP/SAPAP/DAP family of proteins, BEGAIN and MAP1A (3, 4).

Additionally, hDlg binds to several proteins involved in cell cycle regulation and tumorigenesis. It has been recently demonstrated that PKB, a mitotic serine/threonine kinase, interacts with hDlg (5). The APC tumor suppressor forms a complex with hDlg that blocks progression to the S phase of the cell cycle (6). The human papilloma virus E6 and adenovirus E4-ORF1 oncoproteins also interact with hDlg. Furthermore, mutant E6 and E4 lacking sequences required for hDlg binding loose their oncogenic capacity (7, 8).

Several alternatively spliced isoforms of hDlg have been described (Figs. 1 and 6). An alternatively spliced insertion called I1 (1) is located between the unique N-terminal domain of hDlg (NAG) and the first PDZ repeat. The region between the SH3 and GUK domains of hDlg was first characterized as containing two alternatively spliced insertions, I2 and I3 (1). In the same region, a third alternatively spliced insertion, called I4, was described in a brain isoform of hDlg (9). The region of hDlg that separates the insertion sites of I2/I3 and I4 is also alternatively spliced (9). In keeping with our nomenclature, we have termed this region I5.

Only the alternatively spliced insertion I3 has a characterized function. I3 and the PDZ1–2 region of hDlg show similar patterns of charged residues, both forming binding sites for 4.1-like proteins. Both sites contribute to hDlg localization at sites of cell-cell contact (2).

In this study, we identify a novel alternatively spliced insertion, I1B, located in the N-terminal region of the protein. We examine tissue-specific combinations of hDlg alternatively spliced transcripts. We also determine that I1A and I1B form multiple SH3-binding domains, and modulate the level of self-association seen in hDlg. Finally, while it is well documented that I3 is responsible for localization of hDlg to the plasma membrane (2), the role of I2 had not been previously addressed. In this study, we show that I2 is responsible for targeting hDlg to the nucleus.

MATERIALS AND METHODS

PCR Analysis of Alternative Splicing—Select Human Multiple Tissue cDNA libraries (CLONTECH Laboratories) were amplified with primers BL6 and either F5 or C (Fig. 1A). To identify I1A/I1B insertions, four sets of secondary PCR reactions were performed using the primers BL31, BL19, D, and BL14 (Fig. 1A). In separate secondary PCR reactions, I2, I3, I4, and I5 insertions were amplified with primer pairs A + G, A + H, C + F, and A + S, respectively (Fig. 1A). The size of the resulting secondary PCR products were determined by agarose gel electrophoresis. In some instances, PCR products were subcloned and identified by DNA sequencing analysis. To assess the reliability of our analysis, two other sets of tissues cDNA libraries (CLONTECH) were

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§ The abbreviations used are: MAGUK, membrane-associated guanylate kinase; SH3, Src homology domain 3; TBS, Tris-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GUK, guanylate kinase.
used. For each of the three library sets at least three independent experiments were performed and gave consistent results. cDNA Cloning—The sequence of the SH3 domain from hDlg (residues 585–650) was amplified from human B lymphocyte single stranded cDNA. The other cDNA sequences encoding for hDlg polypeptides were PCR amplified from the original hDlg full-length clone (1). Amplified cDNAs were subcloned into the Escherichia coli expression vector pGEX (Amersham Biosciences, Inc.). Preparation of SH3-binding Probes—The purified fusion proteins were dialyzed into phosphate-buffered saline and then mixed with biotin ester at a molar ratio of 1:3 and incubated at 4°C for 2 h. The labeling reaction was quenched with a final concentration of 100 mM Tris, pH 7.5, before dialysis into Tris-buffered saline (TBS).

SH3 Binding Assays—Purified hDlg polypeptides containing the N-terminal region from residues 1 to 220, with or without the I1A/I1B insertions as well as a peptide corresponding to the I1A insertion alone, were resolved by SDS-PAGE and blotted onto nitrocellulose. The blotted proteins were blocked at room temperature for 1 h in TBS supplemented with 0.1% Tween 20, 0.2% gelatin. Avidin was then added to the blocking solution at a final concentration of 1.5 μg/ml and the room temperature incubation was resumed for an additional 1 h. The blots were then further blocked with 1 ml biotin in TBSTG for 1 h. The blots were incubated with the biotinylated SH3 domain probe at a concentration of 1 μg/ml in TBSTG for 4 h at 4°C with constant shaking. The secondary incubation was performed with alkaline phosphatase-conjugated streptavidin (1:5000) in TBSTG for 1 h. The blots were washed four times with TBST between each step. Finally, the blots were developed with nitro blue tetrazolium/4-bromo-4-chloro-3-indolyl phosphate in 150 mM Tris, pH 8.8. These experiments were repeated three times without noticeable differences in the results obtained.

Competition Assays—The synthetic competitor peptide represented the SH3-binding motif from 3BP-1 (Pepl: APTMPPPPLP). In the peptide competition experiments, the biotinylated SH3-Abl probe in TBS-TG was preincubated with a given concentration of Pep1 (25–400 μM) for 2 h at 4°C before being added to the blot. The blot was then processed as described above. In other experiments, unlabeled SH3 domains were used to compete with the biotinylated probes. Specifically, after blocking, the blotted hDlg polypeptides were incubated with the mixed SH3 domains in TBS-TG for 6 h and processed as before. These experiments were repeated three times without noticeable differences in the results obtained.

Cross-linking Experiments—Recombinant proteins in 100 mM Na phosphate buffer, pH 8.3, were incubated 1 h at room temperature in the presence of an increasing concentration of dimethyl pimelimidate-2HCl freshly prepared in 100 mM Na phosphate buffer, pH 8.3. The cross-linking reactions were stopped by the addition of 1/10 volume of quenching solution (50 mM ethanolamine, 20 mM ethylenediamine, in 50 mM Na phosphate buffer, pH 7.4). Samples were either frozen at −80°C until further use or mixed with 5 × SDS-PAGE sample buffer and incubated at 37°C for 1 h. Samples incubated with sample buffer were then resolved by SDS-PAGE using a Tricine gel buffer system. After electrophoresis, proteins were transferred onto nitrocellulose filters. Filters were probed with anti-hDlg antisemur (1:1000) and antigen-antibody complexes were detected with an alkaline phosphatase-conjugated secondary antibody. The blots were developed by incubation with a solution of nitro blue tetrazolium/4-bromo-4-chloro-3-indolyl phosphate. Two distinct batches of proteins were used and identical results were obtained in five sets of cross-linking reactions.

Cell Culture and Immunostaining—The cell line MCF10F (ATCC) was grown at 37°C in 5% CO2 in a humidified incubator in the recommended medium. The cells were grown to ~80% confluence and then fixed in cold methanol. Cells were incubated at 37°C with primary antibodies for 1 h and with specific secondary antibodies at 37°C for 30 min. Alternatively, for control staining, antibodies were first preincubated overnight at 4°C with a 100-fold molar excess of recombinant proteins containing either insertion I2 or I3. The primary antibodies used were the following: affinity purified anti-NAG (diluted 1:100); anti-I2 or anti-I3 (diluted 1:10). In some cases, the cells were stained with 2 μg/ml 4,6-diamidino-2-phenylindole (Molecular Probes). Images were captured using a Zeiss confocal microscope or an Applied Precision deconvolution microscope. In the latter case, Z-series were deconvolved using Delta Vision deconvolution software (Applied Precision Inc.) and shown as maximum intensity quick projections.

Results

Distribution of I1A and I1B Insertions—We have identified a novel 54-base alternatively spliced insertion in hDlg. This insertion, termed I1B, is located between the previously described I1A and I1B insertions. PCR amplification of cDNA molecules was used to analyze all combinations of I1A and I1B insertions found in hDlg transcripts from human brain, skeletal muscle, kidney, liver, cardiac muscle, and lung tissue. In all tissues tested, transcripts containing I1A and I1B together (I1A + B, I1B without I1A (I1BΔA), and neither I1A nor I1B (I1ΔΔ)) were identified (Fig. 2B, Table I). However, consistent with a previous study that found I1A to be barely detectable in brain (9), I1A + B was detected in brain cDNA after a 30-cycle PCR reaction but not after a 20-cycle PCR. This suggested that the transcript coding for I1A + B is present only in low amounts in brain. PCR products containing I1A but lacking I1B (I1AΔB) were not detected in any tests. For this reason, all subsequent PCRs using primer E within I1A were assumed to amplify I1A + B, but not I1AΔB.

The I1A and I1B Insertions Encode an SH3-binding Domain—Based on preliminary experiments showing an interaction between an SH3 domain and full-length hDlg (data not shown) and on the proline-rich character of I1A and I1B (Fig. 2A), the N-terminal portion of hDlg was predicted to contain at least one SH3-binding site. Sequence analysis showed that the
patterns of proline residues contained in these two insertions was similar to the consensus SH3-binding motifs derived from dynamin, mSos1, and 3BP-1 (motifs a, b, and c in Fig. 2A).

The SH3 binding ability of the peptide I1A, and of hDlg polypeptides containing I1A and I1B (N11A/B) or I1B alone (N11B) was tested (Fig. 3). I1A was required for SH3 binding activity (compare Fig. 3, A and B). Preliminary assays showed that the level of biotinylation of each probe was comparable (Fig. 3, C, D, and E), demonstrating a similar demonstration was not possible with the C-terminal

SH3 domain of Crk since its cognate peptide is unknown.

Since hDlg binds to several SH3 domains, we tested the ability of two distinct SH3 domains to bind simultaneously to hDlg. Binding assays using unlabeled SH3-Abl and SH3-Crk as competitors showed no observable competition between these SH3 domains for binding to I1A + B (Fig. 4B), suggesting that both domains interacted simultaneously with distinct portions of hDlg N terminus.

Self-association of hDlg—It has been shown that an hDlg self-association site resides within its N-terminal domain (11). To analyze the impact of I1A and I1B on hDlg self-association, we performed cross-linking experiments on two variants of the N-terminal portion of hDlg, one containing I1A + B and another with I1BΔA. Both variants gave rise to high molecular weight protein bands detected with anti-hDlg antibodies. The major cross-linked product had a size consistent with that of a dimer (Fig. 5). At high concentrations of cross-linker (2 to 2.5 mM), products that might correspond to trimers and tetramers were also observed. A higher level of self-association was ob-
Fig. 4. SH3-Abl and SH3-Crk binding to hDlg. The N-terminal portion of hDlg including the I1A and I1B insertions (NI1AB) was blotted to nitrocellulose and incubated with biotinylated probes. Panel A, biotinylated SH3-Abl was preincubated with varying concentrations of competing peptide Pep1 derived from the SH3 binding sequence of 3BP-1. The effects on SH3 binding of no peptide (lane 1), 25 μM (lane 2), 100 μM (lane 3), and 400 μM (lane 4) are shown. Panel B, NI1AB was probed with biotinylated SH3-Abl or SH3-Crk as indicated at the bottom of the figure. In the case of blots probed with labeled SH3-Abl (lane 1), a parallel strip was first preincubated with unlabeled SH3-Crk (lane 2) at a 10-fold molar excess. Likewise, in the blots probed with labeled SH3-Crk (lane 3), a parallel strip was first preincubated with a 10-fold molar excess of unlabeled SH3-Abl (lane 4).

Fig. 5. Self-association of the N-terminal portion of hDlg. Purified polypeptides corresponding to the N-terminal portion of hDlg and containing the 11B insertion (NI1BAA, left) or I1A and B insertions (NI1AB, right) were incubated with varying concentrations of dimethyl pimelimidate·2HCl. The cross-linked products blotted to nitrocellulose were detected using anti-NAG. The estimated numbers of subunits composing the various cross-linked products are indicated by an equal number of black dots on the right side of each blot.

inserted with NI1BDA than with NI1AB (Fig. 5).

Alternatively Spliced Region between SH3 and GUK—The region comprised between the SH3 and GUK domains contains four alternatively spliced insertions: I2, I3, I4, and I5 (Figs. 1 and 6). PCR analysis was performed on cDNA molecules derived from seven human tissues: brain, placenta, skeletal muscle, kidney, liver, cardiac muscle, lung, and pancreas. I3, I2, and I5 were present in all tissues (Fig. 7, A, B, and D). In contrast, I4 was detected only in brain and liver (Fig. 7C).

The varying patterns of bands observed in I2, I4, or I5 specific amplifications suggested preferential combinations of alternatively spliced insertions in various tissues. PCR reactions using an I2-specific primer produced from one to three products (Fig. 7B). Each of these products was isolated and sequenced; the small (300 bp) product corresponded to I2 alone, and the upper band of the 400-bp doublet corresponded to a combination of I3 and I2. The lower band from the doublet was shown to be a PCR artifact. Sequence analysis of PCR products containing both I3 and I2 revealed a frameshift between the two insertion-coding sequences (Fig. 6B). This shift introduced a stop codon three codons after the end of I3. In vitro translation of hDlg full-length constructs containing I2 and I3 produced a protein truncated immediately after I3 (data not shown).

Sequencing the products of PCR reactions that used an I4-specific primer (Fig. 7C) demonstrated that I4 was always associated with I2 and I5 in the liver. In contrast, I4 was associated either with I2 and I5 (smaller size PCR product) or with I3, I2, and I5 (larger size PCR product) in the brain. In both tissues, the [I2, I4, I5] combination was predominant.

Reactions using an I5-specific primer gave different banding patterns across the tissues tested (Fig. 7D). In order of decreasing size, these bands corresponded to the following combinations of insertions: [I3, I2, I5], [I3, I5], and [I2, I5].

Function of SH3-GUK Alternatively Spliced Region—While the importance of the first two PDZ repeats and the I3 insertion...
for the localization of hDlg to sites of cell-cell contact has been demonstrated previously (2), the impact of the I2 insertion on hDlg intracellular distribution has not. To address this issue, near confluent MCF10F cells were stained with affinity purified anti-NAG antibodies that recognize all hDlg isoforms. In these cells, hDlg labeling was found at sites of cell-cell contact and as discrete domains within nuclei (Fig. 8). A similar distribution was observed with four other cell lines (MCF7, Detroit 551, FHC, and HeLa) and the presence of hDlg within purified nuclei was confirmed by immunoblot (data not shown).

To further characterize the plasma membrane-bound and nuclear hDlg species, MCF10F cells were stained with affinity purified anti-I3 and anti-I2 antibodies. The two types of isoforms displayed distinct staining patterns (Fig. 9). As expected, I3 containing isoforms were localized at sites of cell-cell contact (Fig. 9A). The specificity of the labeling was demonstrated by the significant reduction in membrane staining after preincubation of anti-I3 with a polypeptide containing I3 (Fig. 9B).

Staining with affinity purified anti-I2 antibodies showed that I2 containing isoforms were localized within the nucleus and organized into discrete domains (Fig. 9C). The nuclear staining was eliminated by preincubation of the anti-I2 antibodies with a polypeptide containing I2 (Fig. 9D, arrowheads).

Combination of Insertions from the Two Alternatively Spliced Regions—To identify preferential associations between insertions found at the two sites of alternative splicing, we performed RT-PCR assays using sense primers that specifically amplified one of the three possible N-terminal insertion combinations: I1A + B, I1BAA, or ΔI1. The three types of PCR products were then digested with AvaII, yielding different digestion patterns in the presence or absence of I3 (Figs. 1 and 10). AvaII digestion of PCR products containing only I2 or only I3 will produce a specific 666-bp restriction fragment or two specific fragments (580 and 153 bp), respectively. AvaII digestion of PCR products containing both I3 and I2 will also produce two specific fragments (580 and 189 bp). All three types of PCR products will share the two other AvaII restriction fragments (Fig. 10). The identity of these specific fragments was confirmed by Southern blot using probes specific for either I2 or I3.

Since we focused our analysis on cardiac and skeletal muscles, brain, kidney, and liver tissues and we did not assay for the presence or absence of I4 and I5, the various combinations we identified do not represent the full range of possible isoforms contained in all tissues. Fig. 10 contains an example of the data we obtained using human kidney cDNA. The three types of PCR products derived from the kidney cDNA library gave complex restriction digestion patterns (Fig. 10B, AvaII) indicating multiple combinations of insertions between the two sites of alternative splicing. As demonstrated by Southern blot, kidney isoforms containing I1A + B, I1B alone (I1BAA), or lacking both I1A and B (ΔI1) also contained I2, I3, or I2 + I3 (Fig. 10B, probes I2 and I3). Similar combinations were observed with liver cDNA. In contrast, brain, cardiac muscle, and skeletal muscle contained more limited, yet varied, combinations of insertions (summarized in Table II).

DISCUSSION

It has been suggested that the presence of multiple binding sites within the hDlg protein may allow it to function as a scaffolding protein (2). It stands to reason that isoforms of the hDlg protein containing different combinations of alternatively spliced insertions may in fact have distinct functions within the cell and the presence of particular insertions in hDlg may be of critical importance in cell regulation. Indeed, one study has shown that in two-thirds of neuroblastoma cell lines, the hDlg isoforms present differ from those seen in normal brain tissues (9).

As such, it is important to determine the composition and distribution of isoform combinations of hDlg and to characterize the role of these alternatively spliced insertions. The N-terminal portion of hDlg is characterized by two proline-rich alternatively spliced insertions I1A and I1B (Fig. 2A) that are predicted to form an extended helical domain comprised of up to two polyproline II helices (12, 13). This structural prediction based on the (PXX), consensus together with the general hydroporphic character of the sequence support the hypothesis that the I1A and B insertions are two SH3-binding sites.

We have demonstrated that the I1A and B insertions, in the context of the N terminus of hDlg, form a double binding site that is capable of interacting with several SH3 domains in vitro. Significant binding was detected with both Abl and Crk SH3 domains. The consensus sequence derived from the Abl ligands, 3BP1 and 3BP2, has the form PXKPPPXXP, where the
proline residues at position 1, 6, and 9 are necessary for the binding of 3BP1 to SH3-Abl (14). When the pattern of the essential proline residues is compared with that of motif c overlapping I1A and I1B (Fig. 2A, underlined proline residues), there is a similar sequence in the opposite orientation and the hydrophobic character of the non-proline residues is also conserved. This could account for the binding of SH3-Abl to hDlg, especially since the binding of SH3 ligands is independent of their orientation due to the symmetrical structure of the polyproline II helices. The failure of excess unlabelled SH3-Crk to compete the binding of biotinylated SH3-Abl and vice versa is evidence for the simultaneous association of Crk and Abl with distinct sites.

In light of the well documented promiscuity of binding to polyproline sequences displayed by SH3 domains, it is likely that the I1A and I1B domains can bind a range of SH3-containing proteins. Alternatively, the SH3 domains of the I1A and I1B domains can recruit SH3-containing proteins at sites of cell-cell contact. These proteins may be involved in single or interconnected signaling pathways. Moreover, hDlg self-association increases the ability of this isoform to cluster signaling molecules. The degree of self-association is higher with the isoform lacking I1A although it is less likely to recruit SH3-containing proteins. Therefore, alternative splicing of I1A and I1B in I3 containing isoforms can modulate the function of hDlg isoforms at sites of cell-cell contact.

Sequence comparisons between hDlg and members of the chapsyn family (PSD-93, PSD-95) suggest that if all have a common means of self-association, the cysteine residues found near the N terminus of chapsyns but absent in hDlg are unlikely to contribute to self-association (Fig. 11). This conclusion is supported by the fact that these cysteine residues play a role in PSD-95 and PSD-93 palmitoylation (16, 17). hDlg and chapsyns also share a well conserved 22-amino acid motif (Fig. 11) together with a conserved proline-rich domain corresponding to I1B in hDlg. The contribution of I1B to SH3 binding (Fig. 3) together with overlay experiments showing the self-association of a ΔI1 isoform (data not shown) suggest that I1B is not critical for self-association. Therefore, we propose that the self-association domain is comprised of the 23-residue sequence conserved across hDlg and chapsyns. Searches in data bases using this self-association motif conserved between these sequences in boxed. The cysteine residues (gray highlight) required for PSD-95 and PSD-93 palmitoylation and located at the N-terminal end of these proteins are absent in hDlg.

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Among the three combinations of I1A and B identified by RT-PCR, the isoforms containing I1A + B, which can simultaneously bind to two distinct SH3-containing proteins, were detected in all tested tissues. The isoform predicted to recruit a single SH3-containing protein (I1AΔB) was not detected in any of the eight tissues tested. Isoforms lacking I1A and I1B and those containing only I1B, less likely to bind SH3 domains, were detected in most tissues. The most prevalent hDlg isoform containing I1A, I1B, and I1C3 was detected in all tissues. The isoforms containing I1A and I1B and lacking I1C3 were detected in most tissues. These isoforms containing I1A, I1B, and I1C3 can presumably recruit distinct SH3-containing proteins at sites of cell-cell contact. These proteins may be involved in single or interconnected signaling pathways. Moreover, hDlg self-association increases the ability of this isoform to cluster signaling molecules. The degree of self-association is higher with the isoform lacking I1A although it is less likely to recruit SH3-containing proteins. Therefore, alternative splicing of I1A and I1B in I3 containing isoforms can modulate the function of hDlg isoforms at sites of cell-cell contact.

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Alternatively spliced insertions: I2, I3, I4, and I5 (Figs. 1 and 6). Therefore, it was important to characterize the combinations of these insertions in various tissues. As expected, the [I3, I5] combination was prevalent in most tissues since both I3 and I5 were found in all tissues. Immunoprecipitations with antibodies directed against specific insertions show a good correspondence between the isoform complexity at the transcript and protein levels. Finally, the presence in the same isoform of I2 and I3 was consistent with the previous observation of Mori et al. (9), and such an isoform appears prevalent in skeletal and cardiac muscle. Although transcripts containing both I3 and I2 produced a protein truncated after the I3 region in vitro (data not shown), a regulated ribosomal frameshift leading to a full-length isoform containing I2 and I3 cannot be ruled out in vivo.

We did not study the role of insertions I4 and I5 but instead focused on the impact on I2 and I3 on the intracellular distribution of various isoforms. The membrane localization of the I3-containing isoforms is consistent with the role of I3 in anchoring hDlg to the membrane-associated cortical skeleton through the protein 4.1/ERM protein family and the E-cadherin-catenin adhesion complex (2, 20, 21). As previously described (2), hDlg isoforms were not found in regions of the plasma membrane of cells not engaged in cell-cell contact. The I3 insertion together with the region located between PDZ1 and -2 appear to be solely responsible for hDlg membrane targeting. In that respect, hDlg differs from the members of the chapsyn family, which require either the palmitoylation of their N termini in the case of PSD-95 and PSD-93 or association with heavy metals in the case of SAP-102 for membrane association and subcellular localization in neurons (17). This difference suggests that the association of I3-containing hDlg with the plasma membrane should be more dynamic than that of chapsyns with the plasma membrane at the synapse.

In contrast to the established role of I3, the role of I2 in hDlg nuclear localization remains unclear. Specific nuclear staining with anti-I2 has also been observed in tissue sections suggesting that nuclear distribution is not restricted to established cell lines. Three to five putative nuclear localization signals are predicted in both I2- and I3-containing isoforms, but the I2 insertion itself lacks a canonical nuclear localization signal. Since only I3-containing isoforms are found in the nucleus, it is likely that none of these putative signals are solely responsible for nuclear localization. A yet unidentified I2 binding partner may be responsible for the direct or indirect shuttling or retention of this hDlg isoform into the nucleus. Protein 4.1 is also known to shuttle between the plasma membrane and the nucleus (22). Still, it is unlikely that protein 4.1 is responsible for hDlg nuclear localization since it interacts with I3 and not I2. hDlg is not the only member of the MAGUK family to localize in the nucleus. The protein CASK was recently identified in the nucleus associated with the Tbr-1 transcription factor via its GUK domain (23). Since CASK does not contain a region homologous to I2, we believe that the molecular mechanisms of CASK and hDlg nuclear targeting are different.

Immunostaining also revealed that in cells forming cell-cell contacts, the I2-containing isoforms appeared more evenly distributed between the nucleus and the cytoplasm, and in a few cell clusters, cell-cell contact labeling was observed with anti-I2 antibodies (data not shown). The membrane staining is explained by the ability of the PDZ1–2 motif to interact with members of the ERM family (2). In the absence of quantitative measurements it is difficult to determine whether this distribution change is due to an increasing amount of the cytoplasmic form or a decreasing amount of nuclear hDlg. Nevertheless, this observation shows that the distribution of hDlg isoforms is a dynamic process and may correlate with the physiological state of the cell.

In conclusion, we hypothesize that isoforms containing I1A and -B have a predominant signaling role whereas isoforms lacking I1A and/or I1B have a predominantly structural role. The role of the nuclear form of hDlg has not yet been defined. Based on the function of hDlg at the plasma membrane, it is possible that nuclear hDlg also has a structural role and may nucleate protein complexes at the nuclear matrix. It is noteworthy that hDlg interacts with the mitotic kinase PBK and both proteins are phosphorylated during mitosis (5). This implies that hDlg may be important at various stages of the cell cycle. Indeed, both types of isoforms may function as cell-cycle regulators since mitosis requires both a loss of cell-cell contact during which the I3-containing isoform may play a role, and chromatin reorganization that may involve the I2-containing isoform.

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