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Cloning and characterization of hdlg: The human homologue of the *Drosophila* discs large tumor suppressor binds to protein 4.1

(PSD-95/p55/guanylate kinase/cell–cell contact)

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ABSTRACT **The *Drosophila* discs large tumor suppressor protein, dlg, has been shown to regulate the growth of imaginal discs during embryogenesis [Woods, D. F. & Bryant, P. J. (1991) *Cell* 66, 451–464]. We cloned and sequenced the complete cDNA for a human B-lymphocyte 100-kDa protein that shares 60% amino acid identity with dlg. This human homologue of *Drosophila* discs large (hdlg) contains a C-terminal domain homologous to the known guanylate kinases, a src homology 3 region motif, and three dlg homology repeats. Two nonhomologous domains that can contain in-frame insertions result in at least four alternatively spliced isoforms of hdlg. Several hdlg RNA transcripts are widely distributed in human and murine tissues, and the protein is localized to regions of cell–cell contact. Protein 4.1, the defining member of a family that includes talin and merlin/schwannomin, has the same cellular localization as hdlg, and two sites within hdlg associate *in vitro* with the 30-kDa N-terminal domain of protein 4.1.**

Recessive lesions in seven *Drosophila* genes that have been described as tumor suppressors lead to overgrowth of the imaginal discs. In the lethal (1) discs large 1 (*dlg*) locus, germ-line mutations result in imaginal disc neoplasia and a prolonged larval period followed by death (1). The amino acid sequence of dlg includes several conserved domains: three repetitive sequence motifs containing the sequence GLGF, which have been called dlg homology repeat (DHR) segments, a src homology 3 region (SH3) motif, and a domain that is homologous to the known guanylate kinases (2) (see Fig. 1*A*). These domains, and their organization relative to one another, are conserved across an emerging family of proteins, which include the rat synaptic protein PSD-95, or SAP90 (3, 4), the tight junction proteins ZO-1 and ZO-2 (5–7), and human erythroid p55 (8). We have cloned several isoforms of the human homologue of *Drosophila* dlg (hdlg) from B lymphocytes and present here their sequence features[§] and the general tissue distribution of their RNA transcripts. We also show that both hdlg and protein 4.1 immunolocalize to regions of cell–cell contact and provide *in vitro* evidence for the specific association of the 30-kDa domain of protein 4.1 with two sites on hdlg.

MATERIALS AND METHODS

PCR Screen for hdlg cDNAs. Total RNA was isolated from BM14 B-lymphoblastoid cells using the guanidinium thiocyanate method (9). Single-stranded cDNA was reverse transcribed from 5 μg of RNA after priming with a $(dT)_{17}$+adapter primer as described (10). Each single-stranded cDNA stock was then diluted 100-fold and used in subsequent PCR reactions. We synthesized degenerate primers complementary to four 5-aa stretches flanking two regions of homology between dlg and PSD-95: GLGFN and WWQAR (region A) and GGTDN and NQHIP (region B). Primer sequences were based on the mammalian codon preference: region A primers RPT-1 (sense) GG(N)CT(B)G-G(N)TT(Y)AA(Y)A and RPT-2 (antisense) (N)CG(N)G-C(Y)TGCCACCA; region B primers RPT-3 (sense) G(N)G-G(N)AC(N)GA(Y)AA(Y)CC and RPT-4 (antisense), G(N)G-G(D)AT(R)TG(Y)TG(R)TT (N = G, A, T, or C; B = G, T, or C; Y = T or C; R = G or A; D = G, A, or T). PCR reactions were carried out at low stringency, and products of the predicted size were resolved on agarose gels, excised, and purified before subcloning into a PCR II TA Cloning vector (Invitrogen). The resultant cDNA inserts were sequenced and compared with the analogous region of *dlg*. Out of 24 clones sequenced, 18 showing >65% amino acid identity with dlg were judged positive for the hdlg sequence.

The remaining 5′ and 3′ portions of the hdlg cDNA were cloned using a modified rapid amplification of cDNA ends protocol based on two successive rounds of PCR amplification using nested primers. In the case of the 3′ end, the first round of PCR amplification used the B-lymphocyte $(dT)_{17}$+adapter-primed single-stranded cDNA, probed with primers specific for hdlg and the adapter. The weakly amplified products were resolved on a 3% low-melting-point agarose gel, and the region corresponding to 1–2 kb was excised for use in the secondary PCR reaction with a different pair of nested primers. Products of the secondary PCR were isolated, subcloned, and sequenced as before. All 12 clones sequenced were found to contain the 3′ stop codon of hdlg.

To increase the likelihood of cloning the 5′ portion of hdlg, a selective cDNA minilibrary was reverse transcribed from BM14 RNA primed with a mixture of hdlg-specific oligonucleotides. Replacement synthesis of the second strand was performed as described (11), and the resultant double-stranded cDNA pool was ligated into pBS-SK (Stratagene). The minilibrary was used as the template for two successive rounds of PCR amplification with nested primers as described above. Out of 18 clones sequenced, 5 were found to contain the initiating methionine of hdlg. Analysis of several 5′ and 3′ partial clones revealed sequence insertions in each. Three different insertions, I1, I2, and I3, were isolated.

Northern Blots. Total RNA was isolated from the BM14 B-lymphocyte cell line by the guanidinium thiocyanate method (9), and Northern blots were prepared by standard methods (10). Northern blots derived from multiple human and murine tissues containing 2 μg of poly(A)$^+$ RNA per lane were obtained from Clontech. The blots were probed with a 1150-bp cDNA fragment of hdlg (bases 504–1654) that had been nick-translated with [^{32}P]dCTP (10).

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Abbreviations: hdlg, human homologue of *Drosophila* dlg; GST, glutathione *S*-transferase; GUK, guanylate kinase; SH3, src homology 3 region; DHR, dlg homology repeat.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U13896 and U13897).

Expression of Recombinant hdlg. Plasmid constructs were made that expressed the following as glutathione *S*-transferase (GST) fusion proteins: the entire coding region (accession no. U13897) of hdlg (GST–FL), the N-terminal domain from amino acid 1 to 223 (GST–NT), the three DHR segments from amino acid 201 to 584 (GST–DHR), and the SH3 and guanylate kinase (GUK) domains together from amino acid 584 to 904 (GST–SH3/GUK). The appropriate sequences were PCR amplified from BM14 single-stranded cDNA, and the products were ligated into pGEX-2t (Pharmacia) in-frame for expression as GST fusion proteins in *Escherichia coli*. All constructs were sequenced and produced soluble fusion proteins of the expected sizes. Full-length hdlg constructs bearing I3 alone (GST–FL^{I3}) and SH3/GUK constructs with either I2 (GST–SH3/GUK^{I2}) or I3 (GST–SH3/GUK^{I3}) were isolated.

Generation of Antibodies and Immunofluorescence Microscopy. The hdlg moiety containing all three DHR segments was cleaved from the purified GST–DHR fusion protein using thrombin (12). After further purification on an anion-exchange column, the antigen was used to produce rabbit antibodies as described (13). Anti-hdlg serum (no. 742) was used at a 1:100 dilution to immunostain methanol-fixed MCF-7 human breast carcinoma cells grown on glass coverslips. Cells were also probed with anti-protein 4.1 affinity-purified IgG (no. 14) as described (14). Probes were visualized using a goat anti-rabbit secondary antibody conjugated to fluorescein isothiocyanate and viewed on a Bio-Rad model MRC600 confocal microscope.

Binding Assays with Protein 4.1. Binding assays were performed using 2 μg of GST fusion protein immobilized on 15 μl of packed glutathione beads. In all cases, beads with immobilized protein were incubated with radiolabeled protein 4.1 or its 30-kDa domain in binding buffer [5.0 mM sodium phosphate (pH 7.6), 1.0 mM 2-mercaptoethanol, 0.5 mM EDTA, 120 mM KCl, 0.02% sodium azide, and bovine serum albumin at 1.0 mg/ml; total volume, 50–500 μl]. After incubation for 1–2 hr at 4°C with gentle mixing, the beads were washed five times in 1.5 ml of binding buffer, aspirated to dryness, and either placed in a γ counter (for ^{125}I-labeled 30-kDa domain; triplicate samples) or the bound protein was released in 30 μl of SDS sample buffer for SDS/PAGE followed by autoradiography (for ^{14}C-labeled protein 4.1).

RESULTS

Cloning of hdlg. Since it was unclear whether the human homologue of dlg (hdlg) would be an alternatively spliced form of the p55 transcript or a product of a second distinct gene, we first probed with DNA primers complementary to a conserved region that included the DHR1 segment shared by all of the family members. Out of 24 clones that were sequenced, none corresponded to alternatively spliced transcripts that could account for a dlg-like protein. We then probed by PCR for other conserved regions of dlg not shared by p55: region A (Fig. 1*A*) spanned the middle of DHR3 to the middle of the SH3 motif; region B spanned DHR1 and DHR2. The upstream sense oligonucleotide, which defined the boundary of region A, and the oligonucleotides that defined both boundaries of region B encoded amino acid sequences shared only by dlg and PSD-95/SAP90.

The PCR screen for regions A and B yielded products of the expected sizes. The predicted amino acid sequence of both PCR products was 65% identical to that of the analogous regions in dlg. We then selectively reverse transcribed cDNA from BM14 RNA primed with oligonucleotides specific for the hdlg partial sequence and were able to clone the remainder of the cDNA using a modified rapid amplification of cDNA ends protocol described in *Materials and Methods*.

The hdlg clones were 60% identical to dlg and 70% identical to rat PSD-95/SAP90 on the protein level. The complete sequences have been deposited in GenBank (accession nos. U13896 and U13897). The domain organization characteristic of the other family members was also conserved, with three DHR segments in the N-terminal half of the protein, a central SH3 motif, and a C-terminal GUK domain (Fig. 1*A*). The three DHR segments were between 40% and 53% identical to each other and up to 90% identical to the analogous segments in PSD-95/SAP90. The SH3 motif was most similar to that of dlg with 70% amino acid identity, followed by that of PSD-95/SAP90 and crk. The GUK domain was 40% identical to the entire protein sequence of yeast GUK and 80% identical to the analogous domain in dlg and PSD-95/SAP90. The hdlg GUK domain, like that of dlg and PSD-95/SAP90, had a 3-aa deletion in a glycine-rich motif that is similar to the predicted ATP-binding site of yeast guanylate kinase (15).

Two regions of the hdlg sequence not found in the other family members might represent novel functional domains: a charged, proline-rich 190-aa N-terminal domain (Fig. 1*B*) and a 40-aa internal domain between the SH3 and GUK domains (Fig. 1*C*). When the 5′ and 3′ portions of the hdlg cDNA were PCR amplified from our cDNA minilibrary, both classes of products ran as doublets on agarose gels. Sequence analysis of these PCR products revealed possible insertions in each of the two domains. In the N-terminal domain, either a 99-base insertion (I1) or no insertion occurred after base number 483 (Fig. 1*B*), whereas in the 3′ region either a 36-base (I2) or 102-base (I3) insertion occurred after base 1905 (Fig. 1*C*). The insertions were in-frame, and all of the four possible combinations of 5′ and 3′ insertions were represented among our clones. The I1 insertion in the N-terminal domain encoded a sequence that is particularly proline-rich (10 out of 33) with several tandem sequences similar to the recently derived SH3-binding consensus (16, 17). The I2 insertion was not homologous to other known sequences, while the I3 insertion was 38% identical to the region spanning amino acids 693–727 of dlg, which also lies between the SH3 and GUK domains.

Distribution of hdlg Protein and mRNA. hdlg protein and message were widely distributed in human and murine tissues. Rabbit serum raised against the DHR domain of hdlg, consisting of all three DHR segments, reacted strongly with several proteins around 100 kDa in size and weakly with three proteins between 29 and 42 kDa from human B cells, lung fibroblasts, and cervical epithelia and murine fibroblasts and T cells (Fig. 2). The weakly reactive smaller bands may be due to other proteins with conserved DHR segments or breakdown products of hdlg. No cross-reactivity was observed with erythroid p55.

Northern blots of mRNA isolated from several human tissues all revealed several closely spaced hdlg transcripts whose sizes ranged between 5 and 5.5 kb (Fig. 3). The close spacing of the hybridization signals was consistent with the presence of isoform-dependent sequence insertions. Northern blots of murine tissues revealed a similar pattern of hybridization for multiple isoforms (data not shown).

Immunolocalization of hdlg Protein. The known localization of dlg at septate junctions (2) together with our recent observations that erythroid p55 binds *in vitro* to protein 4.1, a membrane-associated protein (19), raised the possibility that hdlg might be localized to membranes and regions of cell–cell contact. Indeed, the polyclonal antibodies raised against the DHR domain of hdlg specifically stained regions of cell–cell contact in human MCF-7 cells (Fig. 4*A*), and similar membrane associated staining was observed with an antibody to erythroid protein 4.1 (Fig. 4*B*). In both cases, no staining was observed with preimmune serum or anti-hdlg serum preincubated with an excess of hdlg (Fig. 4*C*).

hdlg Binds to Protein 4.1. To determine if the colocalization of hdlg and protein 4.1 represented a direct interaction between the two proteins, various GST–hdlg fusion proteins immobilized on glutathione-agarose beads were incubated

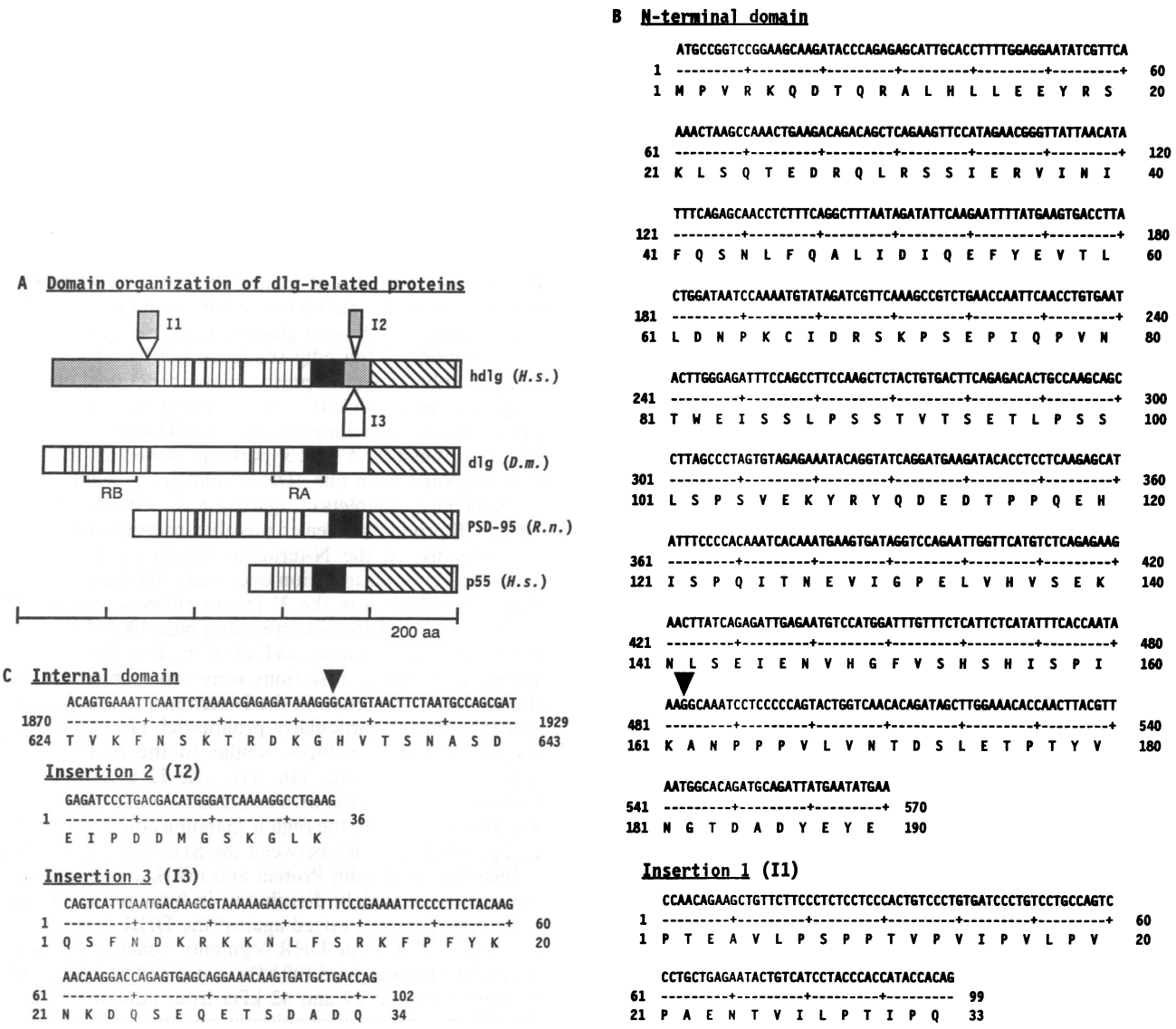

FIG. 1. Sequence domains of hdlg. (*A*) Comparative domain organization of dlg-related proteins. Only the most closely related proteins are shown. The diagonally hatched boxes represent the GUK domains; black boxes, SH3 motifs; vertically striped boxes, DHR segments; and gray boxes, domains and insertions found only in hdlg. The two regions of homology (RA and RB) that were used to design degenerate PCR probes are indicated below dlg. (*B* and *C*) Nucleotide sequence and deduced amino acid sequence of the N-terminal and internal domains found only in hdlg. A black triangle denotes the nucleotide after which at least one isoform insertion has been found to occur. The sequences of the isoform insertion(s) are also given below the domain in which they occur. *H.s., Homo sapiens; D.m., Drosophila melanogaster; R.n., Rattus norvegicus.*

with ^{14}C-labeled protein 4.1 and then sedimented. Both lymphocyte protein 4.1 as well as a 30-kDa labeled breakdown product bound to the beads in an hdlg-dependent manner (Fig. 5, lanes 1–3). Binding to protein 4.1 was mapped to two sites on hdlg: the DHR domain containing all three repeat segments (Fig. 5, lane 4) and the I3 insertion found between the SH3 and GUK domains in a subset of hdlg isoforms (Fig. 5, lanes 5 and 6). Because the N-terminal 30-kDa domain is a well-characterized α-chymotrypsin digestion product of protein 4.1 (21), we confirmed that this domain contains the predominant site that interacts with hdlg at both sites (Table 1). This result is also consistent with our

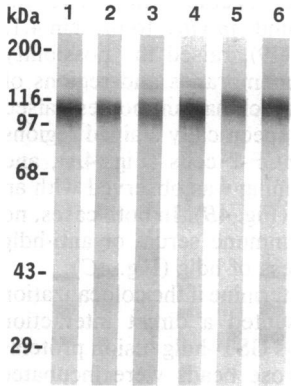

FIG. 2. Western blots of whole-cell lysates probed with anti-hdlg antibodies. Lanes: 1, human BM14 B lymphocytes; 2, HeLa cells; 3, HT29 cells; 4, W138 cells; 5, NIH 3T3 cells; 6, murine T lymphocytes. Whole-cell lysates were resolved by SDS/PAGE, blotted to nitrocellulose, and probed with anti-hdlg polyclonal serum diluted 1:1000 (18). Immunoreactive bands were visualized as described (18) after incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase.

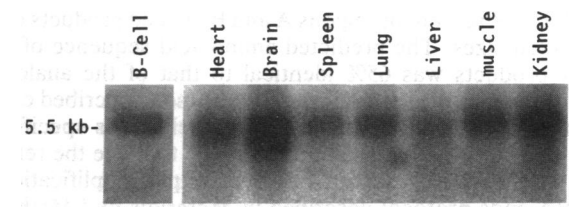

FIG. 3. Northern blots of human mRNA probed with the hdlg cDNA sequence. The tissue source of the blotted RNA is indicated above each lane.

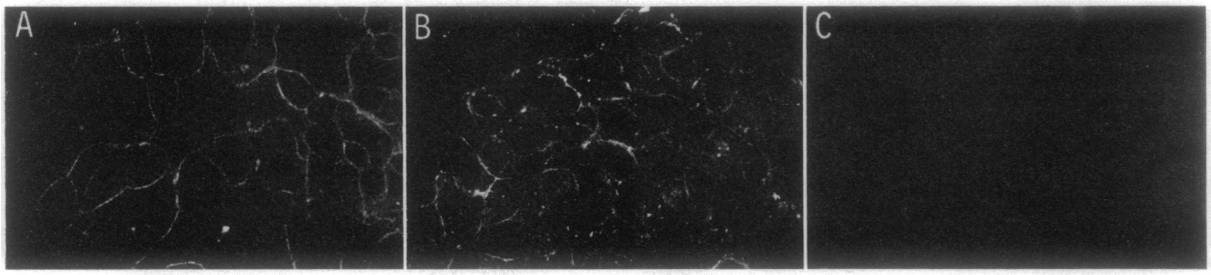

FIG. 4. Immunofluorescence localization of hdlg and protein 4.1 in human MCF-7 cells. Cells were stained with anti-hdlg rabbit immune sera (*A*), with anti-protein 4.1 affinity-purified IgG (*B*), or with anti-hdlg rabbit immune sera preabsorbed against hdlg protein (*C*).

recent finding that the 30-kDa domain of protein 4.1 binds to erythroid p55 within a 38-aa segment flanked by the SH3 and GUK domains (unpublished results).

DISCUSSION

Our results identify the human homologue of *Drosophila* dlg. The hdlg sequence shows that it is a gene distinct from that of p55, and its inclusion in the family of dlg-related proteins provides further evidence that there are two classes of family members: the larger homologues, including hdlg, PSD-95/SAP90, and ZO-1, and the smaller homologues, such as p55. Both hdlg and p55 (24) transcripts are also widely distributed in human and mouse tissues, indicating that large and small dlg-related proteins coexist in many cell types.

All dlg-related proteins share an ≈190-aa domain homologous to the entire length of the yeast GUK enzyme (25), but the large anion pit that is predicted to be the ATP-binding site of GUK (15) is disrupted by a 3-aa deletion in hdlg and the other large family members (26). While the effect of this deletion on the putative enzymatic activity of the dlg homologues has yet to be demonstrated, the GUK domain may not be an active GUK but, instead, a regulatory site that binds guanine nucleotides, as do other G proteins.

A distinctive feature of both hdlg and ZO-1 is the presence of both an SH3 motif and a proline-rich domain in the same protein. The I1 insertion following base 483 encodes a polyproline domain that contains sequences similar to the SH3-binding consensus motifs derived from 3BP-1 (16), mSos1 (27), and dynamin (28) (Fig. 6*A*). Based on the differing specificities observed with the three SH3-binding proteins, the I1 sequence can be divided into several tandem SH3-binding motifs that may bind to more than one class of SH3 domains in a regulated fashion. Furthermore, the $(PXX)_n$ spacing of proline residues is consistent with the formation of an extended polyproline II helix (29), a structure that has been shown to interact with the conserved hydrophobic groove common to the known SH3 structures (30).

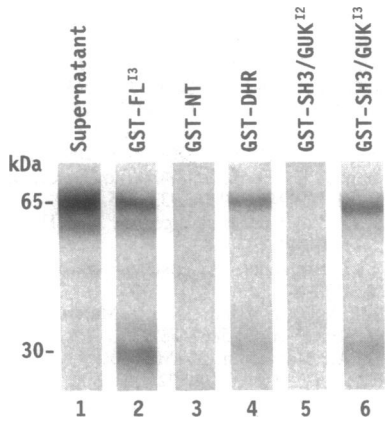

FIG. 5. Association of expressed ^{14}C-labeled protein 4.1 with GST–hdlg fusion proteins immobilized on beads. Autoradiograms of the labeled protein 4.1 in the supernatant (lane 1) and associated with the bead pellet (lanes 2–6) are shown. The immobilized proteins are indicated above each lane. The ^{14}C-labeled protein 4.1 was prepared as follows: A partial protein 4.1 cDNA sequence spanning the second downstream initiating methionine codon to the 3′ end of the reported spectrin binding domain (20) was PCR amplified from the BM14 B-lymphocyte single-stranded cDNA pool described earlier. The PCR product was subcloned into N8, an SP6 eukaryotic expression vector, and expressed in a coupled transcription/translation system (TNT coupled reticulocyte lysate system; Promega) supplemented with [^{14}C]leucine according to the manufacturer's directions.

The hdlg-binding site on protein 4.1 is located within the N-terminal 30-kDa domain, which is conserved in other protein 4.1 family members including talin (31), ezrin (32), the human protein tyrosine phosphatase MEG (33) and the NF2 gene product merlin/schwannomin (34, 35). Protein 4.1 in human erythrocytes links the spectrin network to the membrane via glycophorin C and has been immunolocalized to adherens junctions in epithelial cells (36). Talin is localized at sites of focal adhesion in fibroblasts and at cell–cell contact regions in lymphocytes, perhaps via association with integrins (37, 38). Ezrin, moesin, and radixin have also been immunolocalized to cell–cell adherens junctions and focal adhesion plaques (39). Given that hdlg and protein 4.1 exhibit the same staining pattern at regions of cell–cell contact in MCF-7 cells, hdlg's submembrane localization may be the consequence of interactions with either protein 4.1 or another member of the 4.1 family. Indeed, *Drosophila* protein 4.1 has recently been localized to septate junctions (40) precisely where dlg is located (2), suggesting that the interaction between hdlg and protein 4.1 may be functionally relevant in both vertebrates and invertebrates.

All of the large dlg homologues known contain a DHR domain composed of three 90-aa repeats termed DHR segments. Several proteins contain single DHR segments in-

Table 1. Specific association of the 30-kDa domain of protein 4.1 with the DHR domain and I3 insertion of hdlg

Fusion protein	Bound 30-kDa domain of protein 4.1, cpm
GST–FL^{I3}	1770 ± 19
GST–FL^{I3} + protein 4.1	552 ± 49
GST–NT	269 ± 30
GST–DHR	1452 ± 49
GST–SH3/GUK^{I2}	309 ± 36
GST–SH3/GUK^{I3}	2115 ± 53

Each of the bead-immobilized fusion proteins was preincubated in buffer alone or buffer plus a 10-fold molar excess of protein 4.1, washed, and incubated with identical aliquots of the ^{125}I-labeled 30-kDa domain of protein 4.1 (see *Materials and Methods*). Each value represents the average of three assays ± SD. The ^{125}I-labeled 30-kDa domain was prepared from purified protein 4.1 (22), labeled with ^{125}I-Bolton–Hunter reagent (23), digested to completion with α-chymotrypsin (enzyme to substrate ratio of 1:25), and separated from other fragments on a DEAE-Sephacel column. SDS/PAGE followed by autoradiography showed that >95% of the label was in the 30-kDa domain.

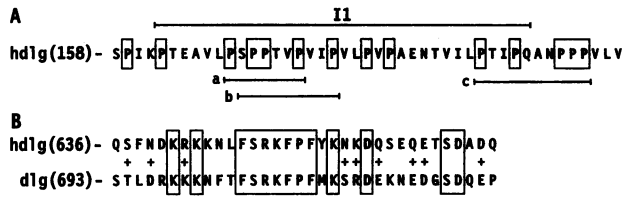

FIG. 6. (*A*) Sequence of the proline-rich region formed by the I1 insertion in the N-terminal domain of hdlg. Proline residues are boxed, and the boundaries of the I1 insertion are bracketed above. Regions that match the proline arrangement found in the SH3-binding motifs of dynamin (a), mSos (b), and 3BP-1 (c) are bracketed below the protein sequence. (*B*) Sequence alignment of the I3 insertion with the analogous region of *Drosophila* dlg. Amino acid identities are boxed, and conservative substitutions are indicated by "+." The first residue position is indicated in parentheses.

cluding p55. Earlier homology searches of the sequence data banks revealed that nitric oxide synthase contains a single DHR segment (3), and we have found that a lymphocyte chemoattractant factor and the dystrophin-associated protein syntrophin (41) also contain DHR segments. Our demonstration that protein 4.1 binds *in vitro* to the hdlg DHR domain provides evidence that this domain functions as a site for protein–protein interaction. Furthermore, preliminary experiments indicate that the hdlg DHR domain associates with at least two proteins in whole cell lysates (data not shown).

Our results also show that a second binding site for protein 4.1 lies between the SH3 and GUK domains of alternatively spliced hdlg isoforms bearing the I3 insertion. The 34-aa long I3 insertion is 38% identical to the analogous region in dlg and may represent another conserved protein-binding domain (Fig. 6*B*). The significance of a distinct subset of hdlg molecules being able to bind protein 4.1 at two sites is unclear, but it is possible that the specificities of the two sites differ, with one having a higher affinity for another protein 4.1-related family member. One site could, for example, facilitate membrane association at regions of cell–cell contact via protein 4.1 while the other is available for interactions with other related proteins. This possibility is consistent with the common subcellular localization of protein 4.1-related family members. Alternatively, a bivalent interaction with protein 4.1 might enable the formation of a physical network of hdlg molecules that could crosslink both protein 4.1 and its associated proteins. The presence of two alternatively spliced protein binding sites, I1 and I3, which bind SH3 domains and protein 4.1, respectively, further suggests that various hdlg isoforms have distinct functions in the cell.

The relationship between negative regulation of cell proliferation and the localization of tumor suppressor proteins at sites of cell–cell contact is not fully understood. In the case of dlg, which localizes at septate junctions, a loss of cell–cell contact in the imaginal discs is an important characteristic of the mutant phenotype (2). Perhaps a causal relationship exists between structural changes in cell–cell contact and the more global event of cell transformation. The evidence we have presented here suggests that dlg-related proteins directly interact with 4.1-related proteins, thus forming a complex between two families of structural and signal-transduction molecules. Indeed, the regulation of this complex at regions of cell–cell contact may dictate how structural proteins contribute to cellular homeostasis.

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