Human Homolog of Disc-large Is Required for Adherens Junction Assembly and Differentiation of Human Intestinal Epithelial Cells*

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Our studies have shown that phosphatidylinositol 3-kinase (PI3K) is recruited to and activated by E-cadherin engagement. This PI3K activation is essential for adherens junction integrity and intestinal epithelial cell differentiation. Here we provide evidence that hDlg, the homolog of disc-large tumor suppressor, is another key regulator of adherens junction integrity and differentiation in mammalian epithelial cells. We report the following. 1) hDlg co-localizes with E-cadherin, but not with ZO-1, at the sites of cell-cell contact in intestinal epithelial cells. 2) Reduction of hDlg expression levels by RNAi in intestinal cells not only severely alters adherens junction integrity but also prevents the recruitment of p85/PI3K to E-cadherin-mediated cell-cell contact and inhibits sucrose-isomaltase gene expression. 3) PI3K and hDlg are associated with E-cadherin in a common macromolecular complex in living differentiating intestinal cells. 4) This interaction requires the association of hDlg with E-cadherin and with Src homology domain 2 domains of the p85/PI3K subunit. 5) Phosphorylation of hDlg on serine and threonine residues prevents its interaction with the p85 Src homology domain 2 in subconfluent cells, whereas phosphorylation of hDlg on tyrosine residues is essential. We conclude that hDlg may be a determinant in E-cadherin-mediated adhesion and signaling in mammalian epithelial cells.

Cadherins mediate cell-cell adhesion through homotypic, calcium-dependent interactions involving their extracellular domains. Classical cadherins have highly conserved cytoplasmic domains that bind α-catenin, β-catenin, and plakoglobin (1). These complexes link actin microfilaments and other integral membrane proteins, allowing the formation of junctional complexes, the organization of the actin cytoskeleton at junctional complexes, the establishment of membrane domains, and the modulation of signal transduction pathways (1–3). In intestinal epithelium renewal, E-cadherin plays a crucial role in the modulation of signal transduction pathways (1–3). In intestinal epithelium renewal, E-cadherin plays a crucial role in the differentiation of intestinal epithelial cells. We report the following. 1) hDlg co-localizes with E-cadherin, but not with ZO-1, at the sites of cell-cell contact in intestinal epithelial cells. 2) Reduction of hDlg expression levels by RNAi in intestinal cells not only severely alters adherens junction integrity but also prevents the recruitment of p85/PI3K to E-cadherin-mediated cell-cell contact and inhibits sucrose-isomaltase gene expression. 3) PI3K and hDlg are associated with E-cadherin in a common macromolecular complex in living differentiating intestinal cells. 4) This interaction requires the association of hDlg with E-cadherin and with Src homology domain 2 domains of the p85/PI3K subunit. 5) Phosphorylation of hDlg on serine and threonine residues prevents its interaction with the p85 Src homology domain 2 in subconfluent cells, whereas phosphorylation of hDlg on tyrosine residues is essential. We conclude that hDlg may be a determinant in E-cadherin-mediated adhesion and signaling in mammalian epithelial cells.

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gesting a role for hDlg in the regulation of cadherin-mediated adhesion in human intestinal cells.

Although a functional role ofDlg/DLG-1 has been suggested in adherens junction formation (7–9), there is currently no evidence supporting this hypothesis in a mammalian system. In the present study, the role and regulation of hDlg in mammalian epithelial cells are investigated. We demonstrate that hDlg associates with several adherens junction proteins in Caco-2/15 cultures, grown on Lab-Tek chamber slides (Nalge Nunc International, Rochester, NY), and was cultured as described previously (14). Secondary antibodies used in immunofluorescence staining were from Chemicon (Mississauga, Ontario, Canada), and antibodies used for Western blotting were from Sigma.

Cell Culture—The Caco-2/15 cell line was obtained from A. Quaroni (Cornell University, Ithaca, NY) and was cultured as described previously (3). HIEC cells were obtained from J. F. Beaulieu (University of Sherbrooke, Sherbrooke, Canada) and were cultured as described previously (14). Primary cultures of human differentiated enterocytes (PCDE) prepared in adherence cultures, grown on Lab-Tek chamber slides (Nalge Nunc International, Mississauga, Ontario, Canada). Antibody raised against cytokertatin-18 was from Sigma. Antibody recognizing actin was purchased from Roche Applied Science. Antibody directed against PI3K p85 regulatory subunit was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-ZO-1 antibody was obtained from Zymed Laboratories Inc. (San Francisco, CA). Secondary antibodies used in immunofluorescence were from Chemicon (Mississauga, Ontario, Canada), and antibodies used for Western blotting were from Sigma.

Immunofluorescence Microscopy—Segments of fetal small intestine and fully differentiated Caco-2/15 cells (30 days post-confluence) were embedded in optimum cutting temperature compound and quickly frozen in liquid nitrogen. Frozen sections of 3 μm were prepared on silane-coated glass slides and air-dried. Sections or PBS-washed Caco-2/15 cell cultures, grown on Lab-Tek chamber slides (Nalge Nunc International, Naperville, IL), were fixed with methanol/acetone (30–70%) for 15 min at −20 °C and blocked with PBS/bovine serum albumin 2% (20 min at room temperature). Samples were immunostained for 1 h with primary antibody and 30 min with secondary antibody. For F-actin staining, fixed cells were incubated with 1 μg/ml FITC-phalloidin for 30 min. Negative controls (no primary antibody) were included in all experiments.

Cell Fractionation Along the Crypt-Villus Axis—Segments of mouse jejunal were inverted onto polyethylene tubing, ligated at both extremities, and washed extensively with KRP buffer, pH 7.5, as described previously (16). Segments were then incubated under agitation with ice-cold isolation buffer (2.5 mM EDTA and 0.25 mM NaCl) for 2 min. After each interval, cell suspensions were centrifuged at 400 × g for 5 min. Pellets were then washed with ice-cold KR buffer and lysed in chilled Triton X-100 lysis buffer (150 mM NaCl, 1 mM EDTA, 40 mM Tris, pH 7.6, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotonin, 0.1 mM orthovanadate, and 40 μg β-glycerophosphate). Cyst or villus origin of the various cell fractions was determined by evaluation of the activities of several brush border enzymes. The specific activity (units/mg of protein) for sucrase-isomaltase (114.10 units/mg in crypt cell preparations; 5323.13 units/mg in villus cell preparations), trehalase (0.04 units/mg in crypt cell preparations; 0.32 units/mg in villus cell preparations), sucrase-isomaltase (0.08 units/mg in crypt cell preparations; 0.17 units/mg in villus cell preparations) was measured as described previously (16, 17).

RT-PCR Analysis—RNA was purified from cultured cells with the Trizol reagent (Invitrogen). The reactions were performed on 1.3–2 μg of purified RNA using the Advantage RT-PCR kit from BD Biosciences Clontech (Palo Alto, CA). Aliquots of the cDNA preparations were submitted to a two-step PCR (13) to identify cDNA sequences coding for known alternatively spliced insertions. Primers F5 and B6 (Fig. 2) were used in the primary PCR. Insertions I2, I3, and I4 were identified using, in the secondary reaction, primer A paired with primers G, H, or F, respectively (Fig. 2). The presence or absence of insertions I1A and I1B was determined by secondary PCRs containing combinations of primers BL14 or BL19 and BL31 or D (Fig. 2). The PCR products were identified as described previously (13).

Dephosphorylation of hDlg by λ and YOP Phosphatases—Caco-2/15 cells were solubilized in Triton lysis buffer, and 600 μg of lystate was immunoprecipitated with hDlg antibodies for 2 h at 4 °C. Beads were washed three times with lysis buffer and twice with the corresponding phosphatase buffer supplied by the manufacturer. The separated proteins were performed in a final volume of 50 μl for 30 min at 30 °C using either λ phosphatase (1000 units; New England Biolabs, Mississauga, Ontario, Canada), YOP phosphatase (2500 units; New England Biolabs), or heat-inactivated phosphatase (65 °C, 1 h). After treatment, the beads were washed again with Triton lysis buffer, and 4× Laemmli’s buffer was added prior to SDS-PAGE and Western blot analysis.

RNA Interference—Double-stranded RNA (siRNA) were designed as described by Donze and Picard (18) and prepared using the Ribomax large scale RNA production system (Promega, Nepean, Ontario, Canada). Sense and anti-sense RNA were treated with RNase-free DNase for 15 min at 37 °C and mixed together. The RNA mix was then heated at 100 °C for 2 min and annealed at 37 °C for 1 h. Targeted sequences began at nucleotides 194, 528, 991, and 1329 of the hDlg-1 gene, respectively (10). Caco-2/15 or HIEC cells were transfected with 50 nm of siRNA using OligofectAMINE (Invitrogen) as recommended by the manufacturer. Cells were fixed at 72 h post-transfection for immuno- fluorescence or lysed for Western blot analysis.

Macromolecular Complex Analysis—Villus-enriched Caco-2/15 cells were washed twice with PBS and treated with 200 μg/ml (in PBS) of DSP cross-linker (Sigma-Aldrich) for 20 min at room temperature. After solubilization in Laemmli’s buffer without β-mercaptoethanol, 75 μg of proteins from untreated and treated cells were separated in duplicate on 5% SDS-PAGE gels. E-cadherin containing macromolecular complexes was visualized by Western blot, and the corresponding region on the duplicate was excised from the gel, reduced, and loaded on top of a 10% SDS-PAGE gel. The separated proteins were analyzed by Western blot.

Far Western Analysis—Cells were washed twice with ice-cold PBS and lysed in chilled Triton lysis buffer, 800 μg (about 100 μl) of cell lysate were boiled in 1% SDS for 5 min (denaturing conditions). Protein samples were subsequently diluted to a concentration of 0.1% SDS, subjected to the immunoprecipitation protocol, separated by SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked for at least 1 h at 25 °C in PBS containing 5% powdered milk and 0.05% Tween 20. Membranes were then incubated overnight at 4 °C with GST-h-cadherin (12) or GST-hDlgSH2 domains (Upstate Biotechnology, Lake Placid, NY) diluted at a concentration of 2 μg/ml in the blocking solution. Membranes were washed 4 times (10 min each) in PBS containing 0.05% Tween 20 and then incubated sequentially with an anti-GST and a horseradish peroxidase-conjugated secondary antibody. Blots were visualized by chemiluminescence.

Dephosphorylation of hDlg by PP1 Phosphatase—Subconfluent Caco-2/15 cells lysates were prepared under the same SDS denaturing conditions used for far Western analysis to eliminate protein-protein interactions. After immunoprecipitation with hDlg antibodies, the beads were treated with PP1 phosphatase (2500 units; New England Biolabs) according to the Promega protocol.

RESULTS

Expression and Localization of hDlg in Human Intestinal Epithelial Cells—Expression of Dlg/hDlg was first analyzed by Western blot in crypt and villus cell populations isolated from adult mouse jejunum and in subconfluent proliferating and...
confluent differentiating Caco-2/15 cells. Caco-2/15 cell line is an intestinal cell line that spontaneously differentiates into a small bowel phenotype with microvilli, apical junctional complex formation, and polarization several days after reaching confluence (3). Biochemical analysis of recovered intestinal epithelial cell preparations revealed relatively pure preparations of poorly differentiated crypt and fully differentiated villus cell populations (see "Experimental Procedures"). Lysates from crypt and villus cell preparations both revealed two major bands of ~110–120 kDa, whereas in fully differentiated villus cells, two additional species with high electrophoretic mobilities were also prominent (Fig. 1A, left panel, see arrows). Expression patterns for hDlg protein in undifferentiated subconfluent and differentiating confluent Caco-2/15 cells were also comparable with that observed in crypt and villus cell populations, respectively, with the exception of an additional band just barely detectable in human confluent Caco-2/15 cells (Fig. 1A, right panel, see arrowhead).

To characterize the intracellular distribution of hDlg in human fetal intestine and to evaluate hDlg localization at E-cadherin-mediated cell-cell contact sites, frozen sections of a 20-week-old human fetal jejunum were labeled with antibodies toward E-cadherin, hDlg, and ZO-1. As illustrated in Fig. 1B, hDlg and E-cadherin stainings were both detected along the lateral cell-cell interfaces throughout the villus axis. The superimposition of both stainings demonstrated a strong co-localization of these proteins in differentiated enterocytes. In contrast, ZO-1 immunoreactivity was restricted to the apex of the lateral membrane of epithelial cells and did not co-localize with hDlg. hDlg expression was also confirmed in Caco-2/15 cells. As reported previously for the parental cell line Caco-2 (12), E-cadherin and hDlg co-localized homogeneously at cell-cell interfaces in 1-day post-confluent Caco-2/15 cells (Fig. 1C). The interaction between E-cadherin and hDlg was confirmed by their co-immunoprecipitation in confluent Caco-2/15 cells (Fig. 1D). Taken together, these results confirm that hDlg co-localizes with E-cadherin in human intestinal epithelial cells.

The Western blot data could suggest that intestinal epithelial cell differentiation is associated with the expression of specific alternatively spliced forms of hDlg. To examine further this hypothesis, hDlg variants were characterized in Caco-2/15 cells. The region of hDlg located between the SH3 and the GUK domains is characterized by four alternatively spliced insertions: I2, I3, I4, and I5 (Fig. 2). Our initial RT-PCR analysis

![Image](http://www.jbc.org/Downloadedfrom)
focused on insertions I2 and I3 whose presence correlates with distinct intracellular localizations of hDlg isoforms (13). The only qualitative difference observed when HIEC, PCDE, and Caco-2/15 cells, collected at various stages of cell density, were analyzed pertained to the presence of a transcript containing insertion I4 in subconfluent Caco-2/15 cells, whereas I4 was not detected once cells reached confluence. I4 was also not detected in either HIEC or PCDE cells (data not shown).

Decreased Phosphorylation of hDlg during Differentiation of Intestinal Epithelial Cells—Because the different protein expression profiles observed in Fig. 1A between undifferentiated and differentiated intestinal epithelial cells cannot be explained by the existence of different alternatively spliced insertions, we investigated whether hDlg phosphorylation could be responsible for this difference. hDlg was immunoprecipitated from subconfluent and confluent Caco-2/15. Beads containing hDlg immune complexes were then incubated with the dual specificity λ phosphatase in order to dephosphorylate the putative phosphorylation sites on hDlg. As shown in Fig. 3A, immunoprecipitated hDlg exhibited two bands in control subconfluent Caco-2/15 cells (left panel), in contrast to five bands in control confluent Caco-2/15 cells (right panel). Interestingly, treatment of hDlg immunoprecipitates with the λ phosphatase revealed five distinct forms of hDlg in subconfluent Caco-2/15 cells (Fig. 3A, left panel, see arrows), including the two forms of lower molecular mass normally observed in confluent cells (right panel). On the other hand, in confluent Caco-2/15 cells, treatment of immunoprecipitated hDlg with phosphatase λ appeared to stabilize the five forms of hDlg (Fig. 3A, right panel, see arrows). Hence, subconfluent Caco-2/15 cells expressed the same variants of hDlg than that observed in confluent cells, but their phosphorylation levels decreased when cells reached confluence and underwent differentiation.

The observed decrease in hDlg phosphorylation when Caco-2/15 cells reach confluence prompted us to further determine whether this decreased phosphorylation was correlated with cell cycle arrest and/or with induction of the differentiation process. Subconfluent Caco-2/15 cells were treated with hydroxyurea, a pharmacological inhibitor of the cellular ribonucleoside reductase, in order to arrest cell cycle in G1/S phase (19), followed by analysis of hDlg protein expression profiles by Western blot. Treatment of subconfluent Caco-2/15 cells with hydroxyurea did induce cell cycle arrest in late G1, as confirmed by the strong inhibition of cell proliferation.2 hDlg expression was not affected however by the hydroxyurea treatment (Fig. 3B, left panel). To analyze further hDlg expression in relation to proliferation, crypt-like HIEC cells were also investigated because these proliferative and undifferentiated intestinal epithelial cells block their proliferation upon reaching confluence but never initiate differentiation (14). As illustrated in Fig. 3B (middle panel), hDlg expression in subconfluent growing HIEC was comparable with that observed in subconfluent Caco-2/15 cells. Of note, hDlg expression was not altered in confluent quiescent HIEC, suggesting that the expression of the fast migrating forms (dephosphorylated forms) is unrelated to the proliferation state of intestinal epithelial cells. However, treatment of immunoprecipitated hDlg with λ phosphatase revealed the fast migrating forms in subconfluent growing HIEC cells, suggesting that HIEC expressed these variants that remained undetected due to their phosphorylated state (Fig. 3B, right panel). Taken together, these results indicate that intestinal epithelial cells express several hDlg variants and that the dephosphorylation of some of these variants is correlated with the initiation of cell differentiation.

hDlg Controls Adherens Junction Integrity—The co-localization of hDlg with E-cadherin in differentiated intestinal epithelial cells further prompted us to determine whether hDlg might control adherens junction integrity. Four synthetic double-stranded siRNAs directed toward different regions of the hDlg gene sequence were transfected into HIEC cells to inhibit hDlg protein expression. As shown in Fig. 4A, cells transfected with siRNAs that target hDlg sequence (10) starting at nucleotides 194 (siRNA/194), 528 (siRNA/528), and 991 (siRNA/991) exhibited strongly reduced hDlg protein synthesis, in contrast to cells transfected with siRNA targeting the hDlg sequence starting at nucleotide 1329 (siRNA/1329). Quantification of three independent experiments indicated that expression of hDlg was reduced by 80% in cells transfected with siRNA/194, siRNA/528, and siRNA/991. siRNA/528 also revealed an unspecified effect as shown by the reduced expression of actin protein in these cells. Nevertheless, the above data indicate that specific inhibition of hDlg protein expression was induced by siRNA/194 and siRNA/991 in intestinal epithelial cells.

These siRNAs were subsequently used to determine whether reduced hDlg expression affects adherens junction assembly and integrity. Because HIEC cells do not form typical junctional complexes when reaching confluence,2 subconfluent Caco-2/15 cells were transfected with siRNA/194. Three days after transfection, cells that had now become confluent were fixed for immunofluorescence and stained for hDlg, E-cadherin, cytokeratin-18, and F-actin with FITC-phalloidin. In contrast to HIEC, Caco-2/15 cells are difficult to transfect. Therefore, we observed a strong reduction in hDlg expression levels in only 5–10% of the cell population (Fig. 4B, panels 1, 3, and 5), an efficiency rate corresponding to the usual transfection efficiency of the Caco-2/15 cell line. As illustrated in Fig. 4B, reduction of hDlg expression by siRNA/194 (panel 1, see arrowheads) decreased the amount of E-cadherin localized at sites of cell-cell contact (panel 2, see arrowheads) in comparison to untransfected cells (panels 1 and 2, see arrows) and cells expressing a control siRNA with a random sequence (data not shown). Furthermore, the decrease of hDlg synthesis is associated with a cytoplasmic distribution of E-cadherin now forming a punctate pattern (Fig. 4B, panel 2, see arrowheads). Cells in which hDlg expression was shut down were comparatively larger in size than untransfected cells (panels 1 and 2), suggesting that loss of hDlg expression caused unstable cell-cell adhesion enhancing cell spreading. In fact, these cells were morphologically similar to subconfluent growing Caco-2/15 cells (Fig. 5A, panels 1 and 2). F-actin organization was also examined following siRNA/hDlg transfection in Caco-2/15 cells. As shown in Fig. 4B, reduction of hDlg expression by RNA interference (panel 3, see arrowheads) led to a disruption and a strong decrease in the amount of F-actin localized at the sites of cell-cell contact (panel 4, arrowheads) in comparison to un-

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2 P. Laprise, A. Viel, and N. Rivard, unpublished data.
transfected cells in which hDlg and F-actin co-localized at cell-cell interfaces (panels 3 and 4, see arrows). However, keratin-18 staining was not altered following siRNA transfection suggesting that intermediate filaments were not affected following loss of hDlg expression (Fig. 4B, panels 5 and 6). The above results suggest that hDlg regulates E-cadherin-mediated cell-cell contact and cortical actin cytoskeleton organization in confluent intestinal epithelial cells.

p85/PI3K Is Associated with hDlg and E-cadherin in Epithelial Cells—We (3) and others (2) have demonstrated recently that E-cadherin engagement recruits and activates PI3K signaling in intestinal and renal epithelial cells. To explore the possibility that PI3K is also associated with hDlg when cells reach confluence, subconfluent (day −3) and post-confluent (day 1) Caco-2/15 and MDCKII cells (data not shown) were double-labeled with antibodies against hDlg and the p85 regulatory subunit of PI3K. In subconfluent Caco-2/15 cells, hDlg was detected at the cell periphery (Fig. 5A, panel 1) whereas p85 staining was restricted to the cytoplasm (Fig. 5A, panel 2) with no significant co-localization observed between hDlg and p85 (Fig. 5A, panel 3). In 1-day post-confluent cells, both hDlg and p85 were localized homogeneously at cell-cell interfaces (Fig. 5A, panels 4 and 5), with superimposition of both staining showing a strong co-localization of these proteins (Fig. 5A, panel 6). Similar results were obtained in MDCKII cells (data not shown). On longitudinal sections, immunostaining of differentiated Caco-2/15 cells (30 days post-confluency) confirmed that hDlg and p85/PI3K are co-localized all along the lateral membranes (Fig. 5B).

The dynamics of hDlg-p85 interaction were further studied in epithelial cells by co-immunoprecipitation assays. Fig. 5C demonstrates that hDlg-p85 association was enhanced and significant in confluent Caco-2/15 in comparison to subconfluent cells. Altogether, these results indicate that p85/PI3K and hDlg are associated at sites of cell-cell contact in confluent epithelial cells.

E-cadherin, hDlg, and p85 Are Part of a Common Macromolecular Complex in Confluent Differentiating Intestinal Epithelial Cells—In an attempt to characterize further the interaction between E-cadherin, hDlg, and p85/PI3K, the cross-linking molecule DSP was used to fix protein-protein interaction in living intestinal cells followed by detection of E-cadherin and E-cadherin-containing complexes by Western blot with the E-cadherin antibody. As illustrated in Fig. 6A, the E-cadherin protein was detected in a major high molecular weight complex following DSP treatment of post-confluent Caco-2/15 cells (see region 2). This E-cadherin-containing band (region 2) was first excised along with region 1 (as a control), after which the cross-linker was subsequently reduced and SDS-PAGE applied in order to separate the proteins present in the complex. Western blot analysis revealed that E-cadherin, p85/PI3K, and hDlg were all recovered from the E-cadherin-containing complex (region 2) but not from region 1 (Fig. 6B, lanes 1 and 2). There was no signal detected with ERK-1 (Fig. 6B), Cdk2, or p27Kip1 antibodies (data not shown), emphasizing the specificity of the DSP approach. These results demonstrate that p85/PI3K and hDlg are associated with E-cadherin in a common macromolecular complex in differentiating intestinal epithelial cells.

p85 Subunit of PI3K Directly Associates with hDlg but Not with E-cadherin—To gain further insight into the mechanism by which PI3K is recruited to E-cadherin-mediated cell-cell contacts, interaction between E-cadherin and the p85/PI3K proteins was analyzed by far Western blotting experiments. Confluent Caco-2/15 cell extracts were submitted to immunoprecipitation by using p85/PI3K antibody or β-catenin antibody (positive control). The components of the immune complexes were resolved by gel electrophoresis and transferred onto a membrane that was probed with GST fused to the cytoplasmic tail of E-cadherin. This fusion protein interacted with β-catenin but did not interact with p85/PI3K (Fig. 7A). These results indicate that the interaction between E-cadherin and p85/PI3K was therefore indirect and may involve intermediate proteins.

In an attempt to verify whether hDlg and p85/PI3K interact directly, far Western blotting experiments were performed using a fusion protein composed of GST in fusion with the N-terminal or C-terminal SH2 domain of p85/PI3K. As illustrated in Fig. 7B (left panel), far Western blots performed with the N-terminal or C-terminal SH2 domain of p85/PI3K revealed two bands on the hDlg immunoprecipitates, whereas no signal was detected on the negative control (precipitation assay performed without antibody). Interestingly, Western blot analysis of hDlg performed on the same membrane demonstrated that these bands corresponded to the fast migrating bands of hDlg (Fig. 7B, right panel), which were only detected in post-confluent differentiating cells (see Fig. 1A). Binding specificity was
demonstrated by the absence of signal when the membrane was probed with GST alone (data not shown).

The direct interaction of both SH2 domains of p85 with hDlg strongly suggested that hDlg was phosphorylated on tyrosine residues. To confirm this hypothesis, hDlg was immunoprecipitated from confluent Caco-2/15 cells, and the hDlg immune complexes were incubated with the YOP tyrosine phosphatase. As shown in Fig. 7C, treatment of hDlg immunoprecipitates with active YOP, but not with heat-inactivated YOP, abolished the association between p85/PI3K and hDlg protein. These results suggest that p85/PI3K binds hDlg through its SH2 domains in a tyrosine phosphorylation-dependent manner.

The above data demonstrated a direct association between p85/PI3K and the fast migrating forms of hDlg, both forms being mostly detected in post-confluent Caco-2/15 and differentiated cells (see Fig. 1A). In addition, p85/PI3K and hDlg both co-localized and co-immunoprecipitated after Caco-2/15 cells reached confluence (see Fig. 5), correlating with the detection of the fast migrating hypophosphorylated forms of hDlg (Fig. 1A). We therefore decided to test the hypothesis that the serine/threonine phosphorylation of hDlg prevents p85/PI3K-hDlg interaction in subconfluent undifferentiated Caco-2/15 cells. hDlg immune complexes purified from subconfluent growing Caco-2/15 were incubated with the serine/threonine phosphatase PP1. Thereafter, far Western blotting experiments were performed using as a probe the fusion protein composed of GST in fusion with the N-terminal SH2 domain of p85/PI3K. Treatment of hDlg immunoprecipitates with PP1, but not with heat-inactivated PP1, revealed five bands for hDlg in subconfluent Caco-2/15 cells including the hypophosphorylated, fast migrating forms (Fig. 7D, right panel). Far Western experiments confirmed that only the fast migrating hDlg forms that are dephosphorylated on Ser and Thr interacted with the N-terminal SH2 domain of p85/PI3K (Fig. 7D, left panel). These results suggest that serine/threonine phosphorylation of hDlg prevents p85/PI3K-hDlg interaction in subconfluent undifferentiated Caco-2/15 cells. hDlg immune complexes purified from subconfluent growing Caco-2/15 were incubated with the serine/threonine phosphatase PP1. Thereafter, Far Western blotting experiments were performed using as a probe the fusion protein composed of GST in fusion with the N-terminal SH2 domain of p85/PI3K. Treatment of hDlg immunoprecipitates with PP1, but not with heat-inactivated PP1, revealed five bands for hDlg in subconfluent Caco-2/15 cells including the hypophosphorylated, fast migrating forms (Fig. 7D, right panel). Far Western experiments confirmed that only the fast migrating hDlg forms that are dephosphorylated on Ser and Thr interacted with the N-terminal SH2 domain of p85/PI3K (Fig. 7D, left panel). These results suggest that serine/threonine phosphorylation of hDlg prevents its association with p85/PI3K in undifferentiated subconfluent Caco-2/15 cells.

hDlg Is Required for the Recruitment of p85/PI3K to E-cadherin

We recently (3) reported that PI3K is necessary for the functional and morphological differentiation of intestinal epithelial cells. Indeed, PI3K activation promotes the assembly of adherens

Fig. 4. hDlg controls adherens junction integrity. A, subconfluent HIEC cells (50% of confluence) were transfected with 50 nM siRNAs that target sequences beginning at nucleotides 194, 528, 991, and 1329 of the hDlg gene. Three days after transfection, cells were lysed and analyzed by Western blot for hDlg and actin expression. B, subconfluent Caco-2/15 cells were transfected with 50 nM siRNA that target sequences beginning at nucleotide 194 of hDlg gene. Three days after transfection (corresponding at day 1 of post-confluence), cells were fixed for immunofluorescence and stained for hDlg (panels 1, 3, and 5), E-cadherin (panel 2), cytokeratin-18 (panel 6), and F-actin with FITC-phalloidin (panel 4). Scale bars, 10 μm.

Fig. 5. p85/PI3K is associated with hDlg at the site of cell-cell contact in epithelial cells. A, subconfluent and 1-day post-confluent Caco-2/15 and MDCKII cells (not shown) were fixed in optimum cutting temperature freezing medium and sectioned longitudinally, fixed, and double-labeled for hDlg and p85/PI3K proteins. Bars, 10 μm. B, fully differentiated Caco-2/15 cells were embedded in optimum cutting temperature freezing medium and sectioned longitudinally, fixed, and double-labeled for hDlg and p85/PI3K proteins. Bars, 10 μm. C, hDlg was immunoprecipitated (Ip) from 600 μg of lysates of subconfluent and 3-day post-confluent Caco-2/15 cells. Immunoprecipitates were solubilized in Laemmli's buffer and were analyzed by Western blotting (WB) to determine the amount of hDlg and p85/PI3K.
junction components with the cytoskeleton. As shown above, hDlg expression is also required for adherens junction and cortical actin cytoskeleton integrity. Moreover, in intestinal epithelial cells, p85/PI3K directly interacts with hDlg in a tripartite complex including E-cadherin. To explore the possibility that PI3K is recruited to cell-cell contact sites in response
**A.**

Fig. 8. hDlg is required for the recruitment of p85/PI3K to E-cadherin-mediated cell-cell contact and sucrase-isomaltase gene expression. A, subconfluent Caco-2/15 cells were transfected with 50 nM siRNA that targets sequences beginning at nucleotide 194 of the hDlg gene. Three days after transfection (corresponding at day 1 of post-conflueny), cells were fixed for immunofluorescence and stained for hDlg (panel 1) and p85/PI3K (panel 2) proteins. Scale bars, 10 μm. B, subconfluent Caco-2/15 cells were transfection with 0.1 μg of sucrase-isomaltase (SI) reporter gene without (control) or with 50 nM of siRNAs which target sequences beginning at nucleotides 194 and 1329 of hDlg gene, respectively, or with an siRNA that targets GFP (negative control). Three days after transfection, luciferase activity was measured. The increase in luciferase activity was calculated relative to control levels (no siRNA) of SI luciferase, which was set at 1. Results are the mean ± S.E. of at least three separate experiments.

**B.**

Adherens junctions form belt-like structures surrounding epithelial cells, thereby allowing cells to adhere to one another to form contiguous sheets (20). Some of the most well studied components of adherens junctions include cadherins and catenins. In a chimeric-transgenic animal model, expression of a dominant-negative N/E-cadherin mutant in villus enterocytes resulted in disruption of cell-cell adhesion associated with an increased enterocyte migration rate along the crypt-villus axis, loss of the differentiated polarized phenotype, and increased apoptosis (4). The E-cadherin-initiated signaling pathways responsible for epithelial homeostasis are only partially characterized. We and others (2, 3) have recently demonstrated that E-cadherin engagement recruits and activates PI3K signaling. We were able to show that this recruitment promotes the assembly of adherens junction components with the cytoskeleton which, in turn, allows the stabilization of the junction and the morphological and functional differentiation of enterocytes (3). Most interesting, PI3K plays a similar role in adherens junction stability, three-dimensional morphogenesis, and tissue-specific gene expression in mammary epithelial cells (21).

The focus of the present study was to evaluate the contribution of hDlg in the formation of adherens junction and enterocyte differentiation. Consistent with the cellular localization of Dlg to lateral junctions, hDlg localizes to regions of cell-cell contact in human epithelial cells (11, 12, this study). Most interesting, in undifferentiated and differentiated intestinal cells, we consistently detected several proteins reacting with antibodies raised against a polypeptide unique to hDlg, which might suggest the expression of alternatively spliced forms of hDlg. Indeed, several alternatively spliced forms of hDlg have been described, and it has been suggested that forms of the hDlg protein containing different combinations of alternatively spliced insertions may have distinct functions within the cells (13). In light of these observations, it was important for us to first determine the composition of hDlg form combinations in undifferentiated and differentiated intestinal epithelial cells. Our data indicate, however, that the hDlg variant expression profiles were qualitatively similar in undifferentiated and differentiated intestinal cells, with the exception of an insertion I4-containing form detected in subconfluent Caco-2/15 cells. I4 was found in a restricted number of tissues, liver and brain, associated with insertions I2 (predominant) or I3 (13), but its function remains unknown. We have demonstrated that the different patterns of immunoreactive proteins observed in Caco-2/15 cells having reached confluency and are in the process of differentiation as well as in differentiated villus enterocytes are rather due to a change in the phosphorylation state of shared hDlg variants (see below).

In epithelial cells, E-cadherin-mediated cell-cell adhesion induces the translocation of hDlg from cytoplasmic pools to the plasma membrane at sites of cell-cell contact (12). In this study, we sought to identify how hDlg might regulate adherens junction in mammalian epithelial cells. Through an RNA interference approach, we demonstrated that loss of hDlg expression in epithelial cells led to a strong decrease in F-actin localized at cell-cell contact sites. The hypothesis that E-cadherin-mediated cell-cell contacts were also affected following reduction of hDlg expression was confirmed by the reduced amount of E-cadherin observed at cell-cell interfaces concomitant with a granular cytoplasmic distribution of E-cadherin. Such cytoplasmic staining for E-cadherin was reported previously in epithelial cells cultured in low Ca²⁺ ion medium resulting in the endocytic uptake of membrane vesicles containing specific con-
constituents of the zona adherens (22). Our findings are consistent with data from other systems demonstrating a disruption of the actin cytoskeleton and alteration in adherens junction assembly and function under conditions in which levels of DLG-1 were reduced (9).

In our opinion, the most interesting and novel finding from this study is that hDlg was also required for the recruitment and maintenance of p85/PI3K to the E-cadherin adhesion complex. Indeed, cells containing a low level of hDlg fail to recruit PI3K to E-cadherin-mediated cell-cell contact and are unable to organize their cortical actin cytoskeleton. PI3K recruitment together with an organized cortical actin cytoskeleton are required for proper adherens junction formation and stabilization and/or morphological and functional enterocyte differentiation (3). Our observation that hDlg, p85/PI3K, and E-cadherin are part of a common macromolecular complex in living epithelial cells supports the hypothesis that, at the adherens junction, PI3K and hDlg may be involved in the signaling events downstream of E-cadherin.

Aside from demonstrating a role for hDlg in the regulation of adherens junction assembly and function, our study also provides new insights into how hDlg recruits PI3K signaling to the E-cadherin adhesion complex. First, the direct interaction between p85/PI3K and hDlg could be mediated by both SH2 domains of p85/PI3K. Accordingly, tyrosine phosphorylation of hDlg was necessary for p85/hDlg association. It has been shown that the binding of both SH2 domains of p85 is required for full activation of PI3K (23) suggesting that the hDlg/p85 interaction may contribute to PI3K activation. Our recent preliminary results demonstrate that hDlg is indeed tyrosine-phosphorylated in undifferentiated and differentiated intestinal epithelial cells. However, the tyrosine kinase(s) that phosphorylate(s) hDlg in intestinal epithelial cells is(are) unknown. Evidence from the literature suggests that p56lck and the epidermal growth factor receptor might be plausible candidates (24, 25). However, our preliminary results demonstrate that the pharmacological inhibition of these kinases did not alter hDlg-p85 association. Hence, an alternative and yet unknown tyrosine kinase is likely responsible for the phosphorylation of hDlg in epithelial cells. Second, far Western analysis demonstrated that the regulatory subunit of PI3K, p85, interacted directly with specific variants of hDlg, and more specifically with the hypo-phosphorylated forms (fast migrating bands) that were clearly present in post-confluent differentiating Caco-2/15 and in isolated villus differentiated cells. Most interesting, the dephosphorylation of hDlg on serine and threonine residues by the PP1 phosphatase clearly revealed an interaction of the SH2 domains of p85 with the fast migrating forms of hDlg, suggesting that in undifferentiated cells serine/threonine phosphorylation of hDlg prevented its association with p85/PI3K. Therefore, it appears from this result that hDlg is already tyrosine-phosphorylated in undifferentiated cells, but the p85-binding site is only unmasked upon differentiation. The change in hDlg phosphorylation state during differentia-
tion to adherens junctions. We propose that, in undiffer-
etiated cells, the pool of pre-existing hDlg molecules associated with E-cadherin is phosphorylated on Ser and Thr and Tyr residues and is maintained in an incompetent state for PI3K recruitment. Once E-cadherin engagement is initiated, the dephosphorylation of hDlg on Ser and Thr residues may result in a change of conformation that renders the phospho-
ylated Tyr residue(s) accessible and promotes the binding of p85/PI3K via its SH2 domains. Upon its recruitment to E-
cadherin via hDlg, PI3K is then activated, allowing for the processes of epithelial cell polarity and differentiation to occur.

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10166

hDlg Links PI3K to E-cadherin

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