Lano, a Novel LAP Protein Directly Connected to MAGUK Proteins in Epithelial Cells*

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Protein networks asymmetrically distributed to basolateral and apical epithelial membranes maintain cell polarity and homeostasis of epithelial tissues. Genetic studies in non-vertebrates assigned two families of basolateral proteins, MAGUK (membrane-associated and guanylate kinase) and LAP (leucine-rich repeats and PDZ) proteins, to a common pathway crucial for the epithelial architecture and acting as a gatekeeper to malignancy. In mammals, three LAP proteins have been described, Densin-180, Erbin, and hScribble. Here, we identify a protein called Lano (LAP and no PDZ) only present in vertebrates and presenting strong identities with LAP proteins. Despite the lack of PDZ domain, Lano is located at the basolateral side of epithelial cells in a similar manner to Erbin and hScribble. Using in vitro and in vivo experiments, we demonstrate that Lano directly interacts with the PDZ domains of MAGUK proteins, including hDLG (human disc large), in epithelial cells. A second pool of Lano is complexed to Erbin. These LAP-MAGUK protein complexes coexist at the basolateral side of epithelial cells. We provide evidence for a direct interaction between LAP and MAGUK proteins, and we propose that various LAP-MAGUK networks targeted to the basolateral side of epithelial cells participate to homeostasis of epithelial tissues and tumor growth.

Multiple defects in signaling pathways participate in the outbreak of carcinoma and target two categories of proteins in epithelial cells. The former are oncogenes such as Myc or the epidermal growth factor receptor (EGFR)1-related tyrosine kinase ERBB2/HER2, which, when overexpressed or mutated, exacerbate a normal function leading to uncontrolled cell proliferation or defects of apoptosis. The latter are products of tumor suppressor genes whose loss of function unleashes a cell regulatory mechanism controlling DNA repair, cell cycling, adhesion, or polarity as in the case of p53 or adenomatous polyposis coli (1). Mutations of oncogenes and tumor suppressors alter the highly organized structure of epithelial tissues maintained by specialized junctions (adherens and tight junctions and desmosomes) and promote loss of cell-cell contacts, cell growth dysregulation, and invasion of healthy tissues by tumor cells (2).

Membrane-associated LAP proteins are recently characterized adaptor proteins important for homeostasis of epithelial tissues and cell transformation. Only present in Eucaryotes, they contain 16 leucine-rich repeats (LRRs), an LAP-specific domain (LAPSD) carboxyl-terminal to the LRR, and either one or four PDZ (PSD95/DLG/ZO-1) domains (see Fig. 1A) (3). Recent genetic analyses have unraveled the role of LAP proteins encoded by scribble in Drosophila melanogaster and let-413 in Caenorhabditis elegans. Both genes are required for proper embryonic development and integrity of epithelial tissues (4, 5). Furthermore, Scribble behaves as a potent tumor suppressor since scribble-deficient flies develop tumors of imaginal discs of epithelial cells (6). Three LAP proteins have been described to date in mammals, i.e. Erbin, Densin-180, and hScribble (3). They share a common modular organization and a basolateral distribution (except for Densin-180, a brain-specific protein) in epithelial cells but bind to a variety of interactors (see Fig. 1A). For example, Erbin binds to ERBB2/HER2 in epithelial cells and neurons and is required for the proper basolateral localization of the receptor (7–9).

More is known about MAGUK, because it was one of the first described PDZ domain protein families. MAGUKs have three PDZ domains, an SH3 domain, and a guanyl kinase domain that interact with a variety of receptors, including the ERBB4/HER4 tyrosine kinase receptor, or cytosolic proteins (NOS and SynGAP) in neurons and epithelial cells (10). In Drosophila, the MAGUK DLG (Disc Large) is crucial for the neuromuscular junction formation but also acts as a tumor suppressor in epithelial cells (11, 12). In humans, hDLG may participate in epithelial carcinogenesis by interacting with adenomatous polyposis coli and impinging on cell cycle events (13, 14).

In Drosophila, a genetic interaction exists between scribble and dlg, because loss of function of either gene gives rise to similar overgrowth of epithelial cells of imaginal discs (6). Interestingly, this MAGUK-LAP connection also exists in nematode, because mutations of let-413 or dlg-1 perturbate very similarly epithelial integrity (15, 16). Although it is doubtless that these two protein families somehow belong to a common

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The abbreviations used are: EGF, epidermal growth factor receptor; LRR, leucine-rich repeat; LAPSD, LAP-specific domain; GST, glutathione S-transferase; HA, hemagglutinin; EST, expressed sequence tag; PBS, phosphate-buffered saline.

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pathway, it remains unclear whether LAP and MAGUK proteins belong to the same protein complexes. The LAPSD is a signature unique to the LAP protein family. In this study, we identified a novel mammalian LAPSD-containing protein we called Lano and ascribed to the LAP family. Like Erbin and hScribble, Lano is located to the basolateral side of epithelial cells where it interacts with MAGUK proteins through a PDZ domain interaction. This interaction is direct and specific as shown by multiple in vivo and in vitro binding assays. Lano can alternatively bind to Erbin in an indirect manner. We show that a direct connection exists between two tumor suppressor families creating protein complexes likely to be important for epithelial homeostasis and cell transformation.

EXPERIMENTAL PROCEDURES

Miscellaneous Procedures—COS-1 cells were grown in Dulbecco’s modified Eagle’s medium containing 100 unit/ml penicillin and 100 μg/ml streptomycin sulfate, supplemented with 10% fetal calf serum. Caco-2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 20% fetal calf serum and 1% non-essential amino acids. All cell transfections were made using Fugene 6 reagent accord-
Two distinct pools of Lano interact with MAGUKs and Erbin. A, two-hybrid in yeast. The baits are the 15-residue carboxyl-terminal peptides of E5B2 (PATAFPYGLDVPV), E5B4 (TVLPYPRHRNTVV), E6 (MSCRESRTRRETQL), Lano (Lano C-term is KNEVNAIDRVTTSV), and Nectin 3 (SHVDGGSVSRREWYY) fused to LexA binding domain. Lano is the full-length protein fused to LexA-BD. Preys are fused to GAL4 activation domain. + stands for growth on the selective -HIS medium and positive B-galactosidase activity. B, non-boiled or boiled (+ SDS) Caco-2 cell lysates were incubated with the mentioned GST-PDZ domains, and bound proteins were detected with anti-Lano antibody (upper and middle panels). The bottom panel shows comparable amounts of recombinant proteins. C, Caco-2 cell lysates were incubated with the mentioned GST fusion proteins. Bound proteins were detected with anti-MAGUK antibody. TL, total lysate. D, schematic representation of Lano interacting directly with MAGUKs and indirectly with Erbin.

**RESULTS AND DISCUSSION**

In searching for new LAPSD-containing proteins in databases, we identified in the human genome several mammalian ESTs encoding a unique protein similar to hScribble and Erbin (3). This new LAP protein contains 16 LRRs and an LAPSD but no PDZ and was thus christened Lano for LAP and no PDZ domain (Fig. 1, A and B). Despite a lack of PDZ domain, we will refer to Lano as an LAP protein, because phylogenetic studies ascribed it to this protein family (data not shown). In comparing full-length LAP proteins, the closest similarity is found with hScribble (60% identity) whereas rat Densin-180 and human Erbin share 42 and 40% identity with Lano, respectively. A lano gene exists in X. laevis and in D. rerio, and they both encode a protein 78 and 75% identical to human Lano, respectively. No Lano was identified in C. elegans and D. melanogaster genomes. The human Lano gene maps to the 6p12.2–12.3 chromosomal band whereas Erbin, Densin-180, and hScribble map on human chromosomes 5, 1, and 8, respectively. A Northern blot analysis showed that Lano mRNA is...
particles, black arrowheads apical membranes (Fig. 1A).

Erbin or hScribble LAP proteins (Fig. 1A) were subjected to immunoprecipitation with pre-immune serum (C) or anti-Lano antibody (anti-Lano or aLano.rec) antibodies, fractioned by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the indicated antibodies. Lano and Ig heavy chains are indicated with asterisks and brackets, respectively (lower panels). Arrowheads indicate hDLG and Erbin in upper panels. C and D, confocal Z sections of Caco-2 cells double-labeled for anti-carcinoma embryonic antigen (CEA) in green (in C) or anti-hDLG antibody in green (in D) and Lano in red (anti-Lano.rec) in C and D. Arrowheads indicate lateral membranes (BL) and apical membranes (AP). E, double immunostaining of Lano (6-nm gold particles, black arrowheads) and hDLG (15-nm gold particles, white arrowhead) in ultrathin cryosections of human colonic epithelium. Lano labeling is observed at the lateral plasma membrane (lpm) and in the cytosol (not shown). Bar = 0.1 μm.

To determine whether Lano interacted directly with MAGUKs and Erbin, we used the two-hybrid system in yeast using PDZ domains as preys and Lano as a bait. In L40 yeast, PSD95 and hDLG PDZ domains interacted with Lano and Lano carboxyl-terminal peptide, as well as to ERBB4/HER4 and E6 control peptides (Fig. 3A) (19–21). No interaction was demonstrated between the Erbin PDZ domain and Lano whereas Erbin bound to an ERBB2/HER2 control peptide (7–9). hScribble and AF6 did not bind to Lano but, as expected, interacted with E6 and Nectin 3, respectively (22, 23). Taken together, these data showed that the MAGUK PDZ domains directly interact with Lano. Failure to evidence the Erbin-Lano interaction by two-hybrid could be because of an indirect binding between these two LAP proteins. To test this possibility, we did a pull-down assay on SDS-treated and boiled Caco-2 lysates to disrupt preformed protein complexes present in cell extracts. This treatment abolished the Erbin-Lano, but not the hDLG-Lano, interaction (Fig. 3B) confirming that Erbin indirectly interacts with Lano in cells. MAGUKs do not bridge Lano to Erbin, because we failed to communoprecipitate Erbin and MAGUKs in epithelial cells (data not shown) and to precipitate MAGUKs with a GST-Erbin PDZ domain (Fig. 3C). These results suggested that a tripartite Lano-MAGUK-Erbin complex is unlikely to exist. We conclude that Erbin and MAGUKs bind to two distinct pools of Lano in epithelial cells (Fig. 3D).

To demonstrate that these interactions occur in vivo, we immunoprecipitated Lano from a Caco-2 cell lysate with two anti-Lano antibodies (see Fig. 1E), and bound proteins were revealed with anti-MAGUK antibody. MAGUK proteins interacted in vivo with Lano (Fig. 4A, upper panel). Reproducing with anti-Lano antibody confirmed that Lano was present in the immunoprecipitates (Fig. 4A, lower panel). Likewise, Erbin commouniprecipitated with Lano in the lysates (Fig. 4B). Thus, both MAGUKs and Erbin bind to Lano in epithelial cells.

dhDLG MAGUK and Erbin are membrane-associated basolateral proteins in epithelial cells (7, 24). To determine the subcellular localization of Lano in epithelial cells, we performed immunostaining and confocal sections of permeabilized Caco-2 cells with anti-Lano antibody. The anti-Lano.rec antibody nicely decorated the basolateral membrane of Caco-2 cells and did not overlap with the immunostaining of anti-carcinoma embryonic antigen, an apical specific marker (Fig. 4C). We used anti-hDLG (clone 2D11) monoclonal and anti-Lano.rec polyclonal antibodies in double immunostainings and observed colocalization between these two proteins at the lateral plasma membrane in Z sections (Fig. 4D). This was confirmed using immunoelectron microscopy on frozen section of human colon. Lano was observed close to the lateral membranes where it

PDZ domains of mammalian LAP proteins are class I domains (Fig. 1A) that interact with membrane receptors containing a carboxyl-terminal (S/T)XV motif (17). Lano contains a carboxyl-terminal TSV motif conserved in vertebrates (zebrafish, xenope, bovine, mouse, and human) matching a canonical class I PDZ domain binding site (Fig. 1A). Interestingly, the Lano carboxyl-terminal sequence (RVTTVS) is very similar to the neurologin carboxyl-terminal motif (HSTTRV), a known target for MAGUK PDZ domains (18). This structural feature prompted us to evaluate whether a biochemical interaction existed between Lano and MAGUK proteins.

To do so, we fused Lano to an HA tag and transiently expressed the protein in COS-1 cells. GST proteins encompassing PDZ domains were used to pull down proteins from lysates. After Western blot using anti-HA antibody, we demonstrated that Lano bound to MAGUK hDLG and PSD95 PDZ domains but not to LIN-2, AF6, Densin-180 (Fig. 2, A and B), and hScribble (not shown) PDZ domains. Similar results were obtained with endogenous Lano from Caco-2 cell lysates (Fig. 2B).

Interestingly, the Erbin PDZ domain also precipitated Lano from the lysates (Fig. 2, A and B). Interaction with PDZ domains was completely abrogated by deleting the three last residues of Lano (ΔLano) to eliminate the PDZ domain binding site (Fig. 2A). We concluded that Lano has a bona fide PDZ domain binding site interacting with MAGUK and Erbin proteins. We also produced GST-Lano and ΔLano and precipitated proteins from a Caco-2 lysate. Bound proteins were revealed by a monoclonal anti-MAGUK protein family antibody that recognizes PSD95, PSD93, hDLG, and Chapsyn-110 MAGUKs. MAGUKs were precipitated by GST-Lano but not by GST-ΔLano (Fig. 2C). We also showed that Erbin, but not hScribble, was precipitated by a GST-Lano recombinant protein (Fig. 2C). Taken together, our results demonstrate that Lano, a new LAP protein, interacts with MAGUKs and Erbin proteins.

Protein Networks Involving LAP and MAGUK Proteins

Fig. 4. In vivo association of Lano and its binding partners at the basolateral side of epithelial cells. A and B, Caco-2 cell lysates were subjected to immunoprecipitation with pre-immune serum (C) or anti-Lano (ΔLano or ΔLano.rec) antibodies, fractioned by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the indicated antibodies. Lano and Ig heavy chains are indicated with asterisks and brackets, respectively (lower panels). Arrowheads indicate hDLG and Erbin in upper panels. C and D, confocal Z sections of Caco-2 cells double-labeled for anti-carcinoma embryonic antigen (CEA) in green (in C) or anti-hDLG antibody in green (in D) and Lano in red (anti-Lano.rec) in C and D. Arrowheads indicate lateral membranes (BL) and apical membranes (AP). E, double immunostaining of Lano (6-nm gold particles, black arrowheads) and hDLG (15-nm gold particles, white arrowhead) in ultrathin cryosections of human colonic epithelium. Lano labeling is observed at the lateral plasma membrane (lpm) and in the cytosol (not shown). Bar = 0.1 μm.
works, we speculate that LAP proteins act as central molecules for a better understanding of the functions of these protein networks. Although we failed to prove an interaction between MAGUKs and Erbin in epithelial cells, a PSD95-Erbin interaction has recently been demonstrated in neurons suggesting that interaction between these proteins may be cell type-dependent (9). Taken together, LAP and MAGUK, two important protein families involved in epithelial homeostasis and cell transformation, form various protein complexes at the lateral side of epithelial cells.

It is interesting to point out that Erbin and MAGUKs bind different subsets of EGFR members in epithelial cells and neurons through PDZ domain interactions (7–9, 19, 20). Although its role remains to be appreciated, Lano may have a dominant-negative effect toward other LAP proteins because of a lack of a protein interaction domain, i.e. the PDZ domain, as shown in other protein families (25). For example, Lano may serve as a regulator by competing with EGFR members for binding to Erbin and hDLG PDZ domains. Hence, a variety of LAP-MAGUK complexes may be linked to mammalian EGFR family members (ERBB2/HER2 with Erbin and ERBB4/HER4 with PSD95) at the basolateral side of epithelial cells and at the post-synaptic membrane in neurons and may regulate their functions. In C. elegans, LET-33, the orthologue of Erbin, is targeted to the basolateral side of vulval epithelial cells by a PDZ domain complex unrelated to MAGUK and LAP (26). Nevertheless, *scribble* and *let-413* genes are crucial for the proper subcellular localization of membrane-associated proteins (4, 5). In mammals, routing or retention of EGFR species at specific subcellular domains of the plasma membrane may rely on LAP-MAGUK networks. Accordingly, Erbin retains ERBB2/HER2 at the basolateral membrane of epithelial cells (7). This idea is strengthened by the genetic link existing between *scribble* or *dlg* and *l(2)gl*, a gene encoding a cortical protein important for vesicle trafficking and protein transport (6, 27–29). Although further studies are necessary to provide a better understanding of the functions of these protein networks, we speculate that LAP proteins act as central molecules in the biological processes mediated by EGFR growth factor receptors. Future studies will also appreciate how, in binding to tumor suppressors (hDLG) and oncogenes (EGFR), LAP proteins may participate to epithelial cell transformation.

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