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The ERBB2/HER2 Receptor Differentially Interacts with ERBIN and PICK1 PSD-95/DLG/ZO-1 Domain Proteins*

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Identification of protein complexes associated with the ERBB2/HER2 receptor may help unravel the mechanisms of its activation and regulation in normal and pathological situations. Interactions between ERBB2/HER2 and Src homology 2 or phosphotyrosine binding domain signaling proteins have been extensively studied. We have identified ERBIN and PICK1 as new binding partners for ERBB2/HER2 that associate with its carboxyl-terminal sequence through a PDZ (PSD-95/DLG/ZO-1) domain. This peptide sequence acts as a dominant retention or targeting basolateral signal for receptors in epithelial cells. ERBIN belongs to the newly described LAP (LRR and PDZ) protein family, whose function is crucial in non vertebrates for epithelial homeostasis. Whereas ERBIN appears to locate ERBB2/HER2 to the basolateral epithelium, PICK1 is thought to be involved in the clustering of receptors. We show here that ERBIN and PICK1 bind to ERBB2/HER2 with different mechanisms, and we propose that these interactions are regulated in cells. Since ERBIN and PICK1 tend to oligomerize, further complexity of protein networks may participate in ERBB2/HER2 functions and specificity.

The *ERBB2/HER2* gene is overexpressed in about 30% of human breast cancers and is also frequently altered in carcinoma of lungs and kidneys. The ERBB2 protein belongs to the large family of receptors with tyrosine kinase activity (RTK)¹ and more precisely to the ERBB subfamily of four receptors which includes the EGF receptor. Upon dimerization and activation, it phosphorylates many substrates including itself. This step induces a network of protein interactions. For example, phosphotyrosine binding and SH2 domains found in cytosolic

proteins such as GRB2 and SHC adaptors bind to phosphorylated ERBB2 and lead to the activation of the RAS pathway (1, 2). RTKs also interact with cytoplasmic proteins in a non-phosphorylated dependent manner by means of PDZ protein modules found in adaptor proteins (3–8).

PDZ domains are 90–100-amino acid domains that interact with short peptides usually found at the carboxyl terminus of proteins. Proteins bearing a S/TXV motif, such as the glutamate receptors (NMDAR) and K⁺ channels, interact with class I PDZ domains found in LIN-7 and PSD-95 (9, 10). The second class of PDZ domains including the LIN-2/CASK PDZ domain prefers a carboxyl-terminal ΨXΨ sequence, where Ψ is a hydrophobic residue. A third class of PDZ domain including the NOS PDZ domain binds to DXV peptides and PDZ domains (11, 12). Within the RTK superfamily, several members have a canonical PDZ domain binding site in carboxyl-terminal position, *i.e.* two ERBB receptors (ERBB2/HER2 and ERBB4/HER4), MUSK (a muscle-specific receptor), and several EPH receptor family members involved in developmental processes such as axon guidance. Kim and colleagues have demonstrated the importance of PDZ domain proteins for RTKs function in nonvertebrates. LET-23, the homolog of ERBB receptors in *C. elegans*, is localized to the basolateral epithelial membrane upon interaction with LIN-7, a PDZ protein that associates with LIN-2 and LIN-10, two other PDZ domain proteins (3, 13). The LIN-7/LIN-2/LIN-10 complex is crucial for proper LET-23 function in insuring a basolateral localization of LET-23 molecules in epithelial cells. Subsequent work has inferred that a subset of mammalian EPH receptors are recruited to specific subcellular compartments in neurons presumably by contacting AF6 and PICK1, two PDZ proteins (4, 5). These adaptors are also targets for the tyrosine kinase activity of EPHR and may participate in their signaling. Among ERBB family members, ERBB4/HER4 is a binding partner for PSD-95, a membrane-associated guanylate kinase also associated with K⁺ channels and NMDAR in neurons (7, 8). It is thought that these PDZ interactions both restrict proteins to specific subcellular compartments and actively participate to signaling events. For example, PSD-95 ^{-/-} mice have an impaired long term potentiation response attributed to a defect in NMDAR signaling (14). Ligands of RTKs including transforming growth factor α , a ligand for EGFR, and ephrins, ligands for EPHR, are also regulated by interacting with GRIP1 and other PDZ proteins including syntenin (15–17).

ERBB2/HER2 contains a *bona fide* PDZ domain binding site in its carboxyl terminus and we previously characterized ERBIN (ERBB2-interacting protein), a novel PDZ domain protein, as a partner for the receptor in epithelia (6). To identify additional ERBB2/HER2 binding partners, we screened a mouse muscle library by the two-hybrid procedure and identi-

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¹ The abbreviations used are: RTK, receptor with tyrosine kinase activity; PDZ, PSD-95/DLG/ZO-1; SH2, Src homology 2; ERBIN, ERBB2-interacting protein; LAP, LRR and PDZ; P75^{NTR}, human neurotrophin receptor P75; MDCK, Madin-Darby canine kidney; GST, glutathione S-transferase; NOS, nitric-oxide synthase; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; 3-AT, 3-aminotriazole; TBS, Tris-buffered saline; BD, binding domain; AD, activation domain; EPHR, ephrin receptor.

fied a second partner for ERBB2/HER2 named PICK1. PICK1 is a single PDZ domain protein originally described as an interactor for PKC α (18). PICK1 is thought to act as a scaffold protein able to cluster receptors and components of signaling or targeting machineries at specific sites in cells (19). We show by using the two-hybrid system, GST pull-down, and coimmunoprecipitation analysis in living cells that PICK1 specifically interacts with ERBB2/HER2. PICK1 binds to the VXV motif found in ERBB2/HER2 but, in contrast to ERBIN, the PDZ domain alone is not sufficient for the interaction. PICK1 also interacts with the VXV motif found in MUSK and ERBB4/HER4 receptors, two RTKs important for the function of neuromuscular junctions, whereas ERBIN prefers a S/T/VXV motif present in ERBB2/HER2 and proteins unrelated to RTK. Furthermore, we show that ERBIN has a propensity to oligomerize through a PDZ-PDZ domain interaction. Activation of ERBB2 interferes with ERBIN, but not with PICK1, ability to interact with the receptor. We finally show that the carboxyl-terminal sequence of ERBB2/HER2 is a dominant basolateral localization sequence *per se* since it is able to redirect an apical receptor to the basolateral surface of MDCK cells. This study suggests that diverse PDZ domain proteins are involved in the regulation of ERBB2/HER2 functions.

EXPERIMENTAL PROCEDURES

Two-hybrid Procedure—To prepare the baits used in this paper, the 15 last amino acids of ERBB2 wild type or mutant or the 15 last amino acids of various proteins were fused to the LexA-BD subunit using pBTM116 vector, which carries *Trp1*. For library screening, an oligo(dT) primed mouse cDNA muscle library cloned in pACT2 vector, which carries *LEU2* as a selection marker, was screened by using the LexA-ERBB2 as a bait and the yeast strain L40 following the lithium-acetate protocol. Approximately 10^6 *TRP*⁺*LEU*⁺ transformants were selected on plates with supplemented minimum medium that lacked tryptophan, leucine, and histidine in the primary screening and contained 10 mM 3-aminotriazole (3-AT) and then tested for the β -galactosidase activity by the filter method in the secondary screening. After rescue, the DNA of selected clones was retransformed in L40 yeast containing LexA-ERBB2 or LexA fused to control peptides. Specific clones were positive for growth in histidine-deficient medium and β -galactosidase activity and encoded for PICK1.

Protein Procedures—Cells were washed twice with cold phosphate-buffered saline and lysed in lysis buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EGTA) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 μ g·ml⁻¹ aprotinin and 10 μ g·ml⁻¹ leupeptin. Sodium orthovanadate at 200 μ M final was added to lysis buffer when cells were stimulated by EGF. After centrifugation at 16,000 \times *g* for 20 min, supernatants were saved for further procedures. For immunoprecipitation, lysates were incubated with antibodies overnight at 4 °C. Protein A-agarose was added and immune complexes bound to beads were recovered after 1 h, washed three times with HNTG buffer (50 mM HEPES, pH 7.5, 10% glycerol, 150 mM NaCl, 0.1% Triton X-100), boiled in 1 \times sample buffer, and separated by SDS-PAGE. Transfer and immunoblotting on nitrocellulose using horseradish peroxidase anti-rabbit or horseradish peroxidase anti-mouse antibody/chemiluminescence method were performed as described (20). For overlay assays, the membrane was incubated 2 h at room temperature with soluble GST fusion proteins labeled with protein kinase A and [γ -³²P]ATP diluted in TBS, 5% dried milk, 1 mM dithiothreitol (10⁶ cpm/ml). After rinsing with TBS, 0.1% Triton X-100 and TBS buffers, bound GST was revealed by autoradiography. GST production and GST binding assays were performed as described previously (20).

Cell Culture—COS-1 and MDCK II cells were grown in Dulbecco's modified Eagle's medium containing 100 units·ml⁻¹ penicillin and 100 μ g·ml⁻¹ streptomycin sulfate, supplemented with 10% fetal calf serum. All cell transfections were made using Fugene 6 reagent according to manufacturer's recommendations (Roche Molecular Biochemicals).

DNA Constructs—Human *ERBIN* cDNA was used to create different constructs allowing expression of the protein in procaryote or eucaryote cells (GST, Myc, LexA-BD-tagged proteins). The RK5-myc vector was used to express proteins fused to the Myc epitope (20). The pGEX-Tag vector was used to produce all GST fusion proteins. Site-directed mutagenesis were performed using the Quick-Change kit (Stratagene). All

constructs were sequenced by Genome Express, SA (Grenoble, France). A rat cDNA library was used as a template to amplify a fragment encoding the DENSIN-180 PDZ domain (residues 1342–1492) and AF6 PDZ domain (residues 1007–1124).

Chimeras between the human P75NTR and human ERBB2/HER2 or GBT-1 were done by PCR using reverse primers coding for the last 15 amino acids of either ERBB2/HER2 or GBT-1 in order to replace the last 15 residues of P75NTR. All constructs were sequenced and subcloned into pIRES (CLONTECH). The resulting plasmids were transfected into MDCK II cells, and stable populations were obtained after G418 selection.

Antibodies—Anti-Myc 9E10 (Oncogene Research Products, Cambridge, MA) monoclonal antibody was used for immunoprecipitation and immunoblotting. 4G10 (anti-PY) monoclonal antibody was from Upstate Biotechnology Inc. Goat anti-rabbit and anti-mouse IgG coupled to horseradish peroxidase were purchased from Jackson Laboratory and Dako, respectively. A rabbit anti-ERBIN polyclonal antibody was produced by injecting a soluble GST-ERBIN-(914–1371) fusion protein. Anti-PICK1 goat antibody was from Santa Cruz Inc.

Immunofluorescence and Cell Surface Biotinylation—Transfected MDCK cells were grown on coverslips for 3 days after confluence and then treated as described (21) using ME 20-4, a monoclonal antibody against the extracellular domain of human P75NTR and a rabbit polyclonal antibody against Gp114, an apical glycoprotein of MDCK II cells (22).

Transfected MDCK II cells were grown on Transwell® filters for 5 days after seeding and processed for selective cell surface biotinylation as described before (23). P75NTR or chimeras were immunoprecipitated using the ME 20-4 antibody followed by streptavidin precipitation. Quantitative analysis was done using the Bio-Image IQ software.

RESULTS

Identification of Residues in ERBB2/HER2 Important for ERBIN/ERBB2 Interaction—We have recently isolated ERBIN, a partner for ERBB2/HER2 in epithelia. ERBIN is a novel PDZ domain protein belonging to the LAP (LRR and PDZ) protein family that includes DENSIN-180, SCRIBBLE, and LET-413 (6, 24–27). The ERBIN PDZ domain interacts with the 15 carboxyl-terminal residues in ERBB2, which contain a VXV motif. We used the two-hybrid system in yeast to further delineate critical residues important for this interaction. Position for each residue is given starting position (0) for the carboxyl-terminal valine, followed by (–1) for the proline, (–2) for the valine, and so on (Fig. 1A). Each amino acid in the ERBB2 peptide was changed to alanine except for alanine in –12 position changed to glycine. Peptides fused to the LexA binding domain (LexA-BD) were challenged against the GAL4 activation domain (GAL4-AD) fused to the ERBIN PDZ domain (Fig. 1A). Interaction was evidenced by the capacity of cotransformed L40 yeast to grow on plates depleted of histidine (–*HIS*) supplemented with 10 mM 3-AT and to present a positive β -galactosidase activity. As previously shown, mutation of the carboxyl-terminal valine (mutant VA) and deletion of the 6 last residues (data not shown) in ERBB2 abrogated the interaction with ERBIN (6). Interaction was also altered when Val in –2 position, Asp in –3 position, Leu in –4 position, and Tyr in –7 position were mutated (Fig. 1A). We expressed ERBB2 (wild type and mutants) in COS cells and precipitated receptors with a GST-ERBIN PDZ domain in a pull-down assay (Fig. 1B). Replacement of the COOH-terminal valine, aspartic acid in –3 position, leucine in –4 position, and tyrosine in –7 position to alanine as well as truncation of ERBB2 (Δ 6 mutant) reduced the binding to the ERBIN PDZ domain. No change was found when asparagine in –10 position was mutated to alanine. Mutation of valine (–2) did not modify the interaction in the pull-down assay contrasting with the two-hybrid data. This may imply that this residue is not as crucial for ERBIN-ERBB2 interaction *in vivo*. To obtain further information on the ERBIN PDZ domain binding specificity, we used a Far Western strategy to test the interaction of this domain with peptides found in the carboxyl terminus of receptors and ion channels (Fig. 1C). These sequences, except for EGFR and ERBB3, are

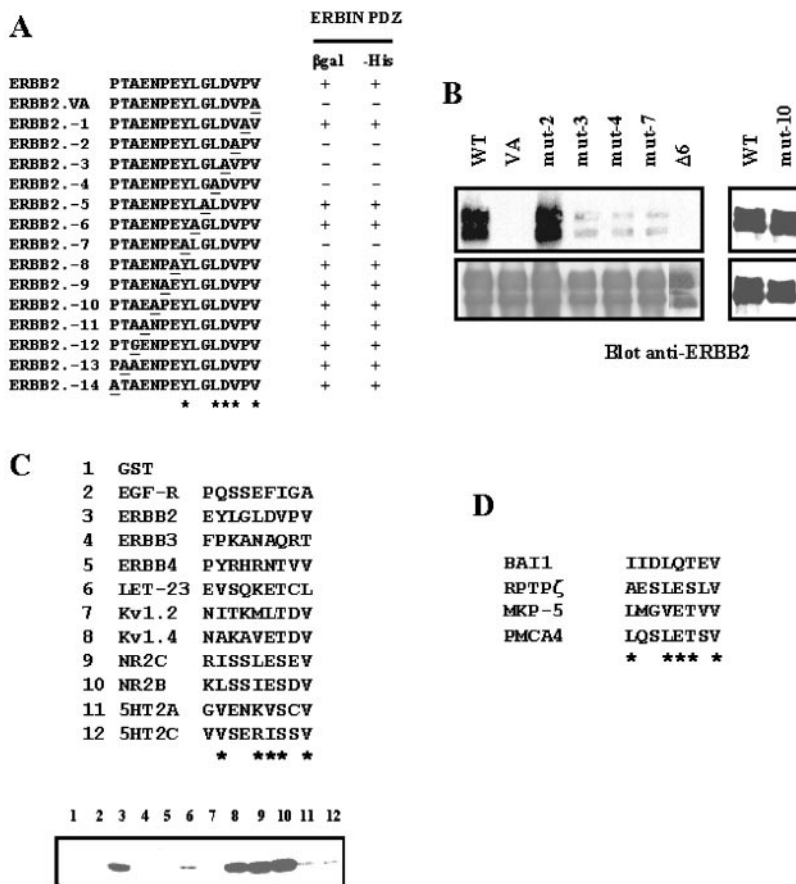


FIG. 1. Binding specificity of the ERBIN PDZ domain. A, the last 15 carboxyl-terminal amino acids found in ERBB2 (wild type or mutants) were fused to the LexA-BD and co-transformed in L40 yeast with GAL4-AD ERBIN (1240–1371) referred as the ERBIN PDZ domain. Co-transformed yeast were plated on $-\text{Trp-Leu-His}$ medium containing 10 mM 3-AT. + means growth on the selective medium ($-\text{His}$) and positive β -galactosidase activity (β gal). Asterisks point to the residues important for ERBB2/ERBIN interaction. Mutated residues are underlined. B, HER1/2 (chimera EGF-R/ERBB2) wild type (WT) and mutant were transiently expressed in COS cells and pulled down with GST-ERBIN PDZ domain. Bound receptors were revealed with anti-ERBB2 antibody after Western blot (upper panel). Mutation of the carboxyl-terminal valine (VA), aspartic residue in -3 position (*mut-3*), leucine in -4 position (*mut-4*), tyrosine in -7 position (*mut-7*), and deletion of the last 6 residues in ERBB2 ($\Delta 6$) abrogate the ERBB2/ERBIN interaction. Lysates were run, transferred on nitrocellulose, and probed with anti-ERBB2 to show a comparable expression of receptors (lower panel). C, direct interaction between the ERBIN PDZ domain and ERBB2, Kv1.4, NR2C, and NR2B peptides. The last 9 amino acids of the mentioned proteins were fused to the GST protein, Western-blotted, and probed with soluble ^{32}P -labeled GST-ERBIN PDZ domain or GST (data not shown) fusion proteins. Bound proteins were revealed by autoradiography. No binding was found with GST alone (data not shown). Red Ponceau staining showed that similar amounts of GST proteins were loaded (data not shown). D, sequence of the carboxyl terminus of four putative ERBIN-interacting proteins.

known ligands for PDZ domains. Nine-amino acid peptides were fused to the GST protein, and equivalent amount of proteins were resolved by Western blot. The membrane was incubated with a soluble and radiolabeled ^{32}P -GST-ERBIN PDZ. After washing, the membrane was exposed for autoradiography to detect bound radiolabeled proteins. No signal was found when we used a radiolabeled GST alone or GST-AF6 PDZ domain as a probe (data not shown). As expected, the ERBIN PDZ domain interacted with ERBB2 but not with other human EGF family members while ^{32}P -GST-LIN-7 efficiently bound to the worm LET-23 (data not shown). Among the other fusion proteins tested, the carboxyl termini of Kv1.4, NR2C and NR2B interacted strongly with ERBIN (Fig. 1C). Interestingly, these peptides have sequence similarities to the ERBB2 peptide at the crucial positions found for ERBIN interaction, namely a carboxyl-terminal valine, an acidic residue in -3 position (Asp or Glu), and hydrophobic residues in -4 and -7 positions (tyrosine, isoleucine, or leucine). Position -2 can support a serine, threonine, or valine. Taken together, these data demonstrate that the ERBIN PDZ domain binds to a $\Psi\text{XX}\Psi[\text{ED}][\text{STV}]\text{XV}$ peptide, where Ψ represents hydrophobic residue and X any amino acid. We searched in data bases for other mammalian proteins containing such a motif and found

the proteins listed in Fig. 1D, including a transmembrane phosphatase (RPTP ζ), PMCA4, a calcium pump, and MKP5, a dual specificity phosphatase specific for p38 and stress-activated protein kinase/c-Jun NH $_2$ -terminal kinase. These are potential binding partners for ERBIN. Interestingly, brain-specific angiogenesis inhibitor 1 (BAI1), a potential ERBIN interactor with seven-span transmembrane domains, has indeed been shown to bind PDZ domain proteins (28).

Identification of Mutations within the ERBIN PDZ Domain That Modify the ERBB2/ERBIN Interaction—X-ray crystallographic studies of PSD-95, DLG, and CASK/LIN-2 show that PDZ domains comprise two α helices and six β sheets (29–31). The COOH-terminal peptide of receptors binds to the groove between the second α helix and the second β sheet with a GLGF motif providing a carboxylate-binding loop. A residue found in the second α helix (His for class I; Val, Leu, or Gln for class II; and Tyr for NOS class III PDZ domain) selects a certain type of ligand depending on the residue found in -2 position in the peptide. Hence, the PSD-95 class I PDZ domain prefers a Ser or Thr in -2 position in the peptide ligand and the CASK/LIN-2 class II PDZ domain selects hydrophobic residues in -2 position while class III PDZ domain in NOS binds to an aspartic residue (Asp) in -2 position. In a previous report, we identified

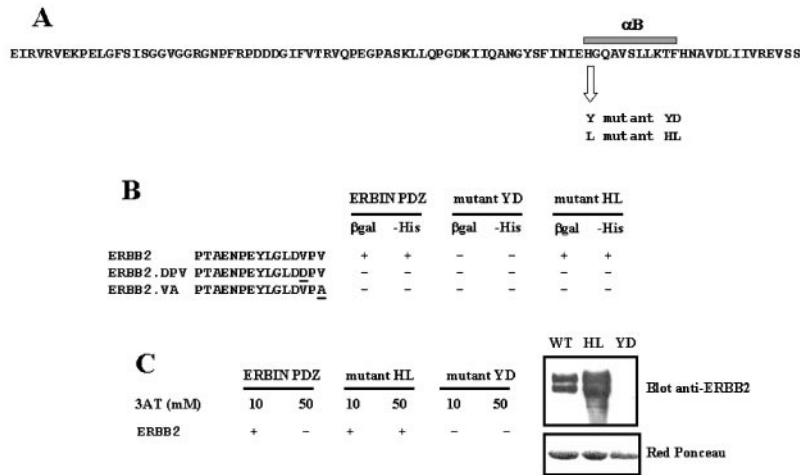


FIG. 2. Mutations within the ERBIN PDZ domain modulate the ERBB2/ERBIN interaction. *A*, sequence of the mouse ERBIN PDZ domain. An arrow points to a conserved histidine found in the α B helix of class I PDZ domains but not in NOS and class II PDZ domains (data not shown). Mutations of His-Gly to Tyr-Asp as in NOS (mutant YD) and His to Leu as in class II PDZ domains (mutant HL) were engineered by site-directed mutagenesis. *B*, same as in Fig. 1*A*. Effects of mutations within ERBB2 and the ERBIN PDZ domain were tested by two-hybrid in yeast. *C*, in the left panel, the experiment is same as panel *B* using increasing amount of 3-aminotriazole. In the right panel, a pull-down assay was performed on HER1/2 expressing lysates with GST-ERBIN PDZ wild type (WT) and mutant (HL and YD). The interaction with HER1/2 was revealed by anti-ERBB2 antibody. Ponceau Red staining shows that equal amounts of GST proteins were used.

a mutation within the ERBIN PDZ domain (mutant YD) changing the His residue in α B helix to Tyr as found in NOS, which abrogates the interaction with ERBB2 (6). The NOS PDZ domain interacts with DXV motifs. We mutated the Val-2 residue to Asp-2 in ERBB2 but no interaction was found with the ERBIN YD mutant by using the two-hybrid system (Fig. 2*A*). Thus, mutation YD abrogates interaction with ERBB2 but does not shift the ERBIN PDZ domain specificity from class I to class III. This result can be explained by the fact that the functional NOS PDZ domain requires additional amino acids beyond the conserved PDZ domain consensus domain (12). This additional region is not present in the ERBIN PDZ domain. We also mutated the histidine to leucine as found in class II PDZ domain (mutant HL). Surprisingly, interaction with ERBB2 was improved as L40 yeast co-expressing LexA-ERBB2/GAL4-ERBIN HL grew on -HIS plates containing 50 mM 3-AT in contrast to LexA-ERBB2/GAL4-ERBIN PDZ domain wild type. Growth was identical on 10 mM 3-AT plates. This result was confirmed in a GST pull down assay where ERBB2 was more efficiently precipitated by ERBIN HL mutant than by ERBIN wild type (Fig. 2*C*). The VXV motif in ERBB2 binds to the class I ERBIN PDZ domain, although it conforms to the Ψ X Ψ motif binding site for class II PDZ domains. Changing His to Leu in ERBIN PDZ domain α B helix improves the binding to VXV-containing peptides. Accordingly, the ERBIN PDZ domain mutant HL now binds to the VXV motif found in EPHB2 (data not shown). We have thus identified important residues within ERBB2 and ERBIN peptide sequences responsible for the interaction between the PDZ domain and its ligand.

PICK1, a New Partner for ERBB2/HER2 Receptor—Promiscuous interaction with various receptors is a frequent theme for PDZ domains. For example, the CASK/LIN-2 PDZ domain binds to neuroligins and syndecans (32–34) and PSD-95 interacts with glutamate receptors, K^+ channels, and adenomatous polyposis coli (35–37). On the other hand, receptors can bind different PDZ proteins, e.g. a class of EPH receptors binds to syntenin and AF6 PDZ domains (4, 5). We have shown that the ERBIN PDZ domain interacts *in vitro* with several peptide ligands (Fig. 1*C*). Conversely, it is likely that ERBB2 binds to other PDZ domain-containing proteins. To search for other ERBB2 partners, we screened a murine muscle cDNA library with LexA-ERBB2 as a bait. Four different clones were pulled

out, each encoding the full-length PICK1 protein (Fig. 3*A*). PICK1 is an ubiquitous 50-kDa protein containing an amino-terminal PDZ domain and a coiled-coil region in its carboxyl terminus. PICK1 was originally described as a partner for PKC α , AMPA, EPH receptors, and, more recently, water channel aquaporins through a PDZ domain interaction (5, 18, 19, 38). We found no binding between PICK1 (or ERBIN) and ERBB2 when the carboxyl-terminal valine in ERBB2 was replaced by an alanine (LexA-ERBB2.VA) (Fig. 3*A*). To further delineate the region of PICK1 involved in the interaction, we introduced a 100-amino acid carboxyl-terminal deletion in the protein and failed to detect an interaction between deleted PICK1(1–305) and ERBB2. Additionally, a previously described mutation within the PICK1 PDZ domain changing K²⁷D²⁸ to AA also disrupted the interaction PICK1-ERBB2 (Fig. 3*A*) (39). As in the case of NOS PDZ domain, these results suggest that the PICK1 PDZ domain is necessary but not sufficient to bind to ERBB2 (12).

To confirm our two-hybrid data, we produced GST fusion proteins encompassing PICK1 (GST-PICK1), deleted PICK1 (GST-PICK1 1–305), mutated PICK1 (GST-PICK1mut), and the PICK1 PDZ domain (GST-PICK1 PDZ). COS cells were transfected with pCMV-ERBB2, and cell extracts were incubated with GST proteins bound to glutathione-agarose beads. After washing, proteins were resolved on SDS-PAGE and transferred on nitrocellulose. Probing of the membrane with anti-ERBB2 antibody showed that only GST-PICK1 bound to ERBB2, although equivalent amounts of all GST fusion proteins were present on beads (data not shown). As expected, GST-ERBIN PDZ domain pulled down ERBB2 from the lysate (Fig. 3*B*). An identical result was found when we performed a pull-down assay on ERBB2 extracted from MDCK cells (data not shown).

PICK1 has been described as a binding partner for other proteins than ERBB2. We thus asked whether ERBIN interacts with known PICK1 ligands including MUSK, EPHB2, and PKC α (5, 39). The carboxyl-terminal peptide sequences of these proteins were fused to LexA-BD and coexpressed with GAL4-AD-ERBIN PDZ domain or PICK1 in L40 yeast (Fig. 3*C*). We additionally challenged ERBB4 and PDGFR α and - β with the two PDZ domains. In contrast to ERBIN, which interacted solely with ERBB2, PICK1 also bound to ERBB4, MUSK,

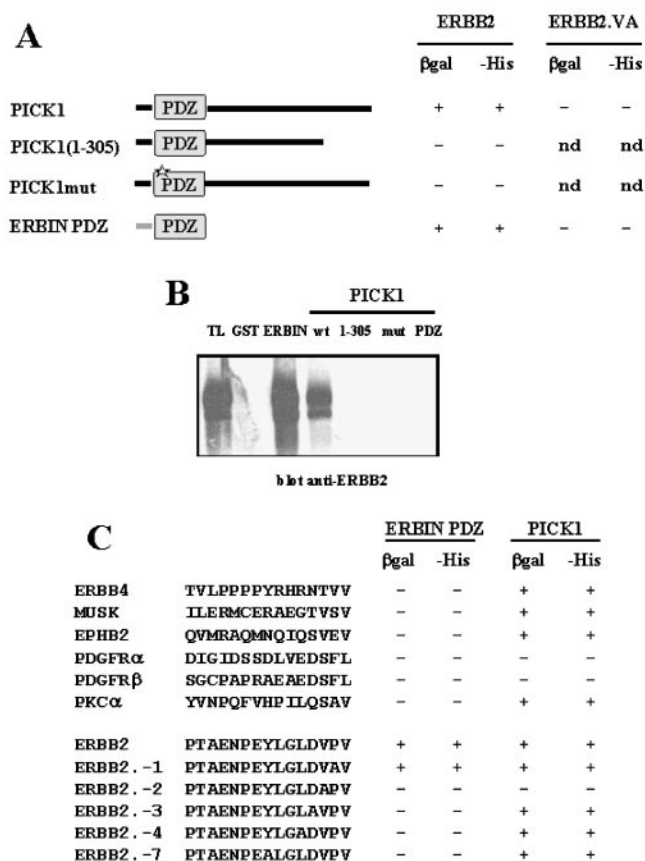


FIG. 3. PICK1 is a new binding partner for ERBB2/HER2 receptor. A, full-length mouse PICK1 was pulled out by screening a muscle cDNA library by two-hybrid using LexA-BD-ERBB2 as a bait. GAL4-AD-PICK1 wild type and mutants were challenged against LexA-BD-ERBB2 or LexA-BD-ERBB2.VA mutant. Truncation (PICK1(1–305)) or mutation within the PICK1 PDZ domain (mutation of K²⁷D²⁸ to AA) abrogate the binding with ERBB2. GAL4-AD-ERBIN PDZ domain was used as control. *nd*, not determined. B, HER1/2 was transiently expressed in COS cells and pulled down with the mentioned GST fusion proteins. After washing, bound receptors were probed with anti-ERBB2 antibody. C, same as Fig. 1A using carboxyl-terminal sequences of the mentioned proteins fused to LexA-BD.

EPHB2, and PKC α peptides. A panel of ERBB2 mutants was coexpressed with GAL4-AD-PICK1 or ERBIN PDZ domain. The ERBB2-PICK1 interaction was abrogated when the carboxyl-terminal motif VXV was mutated but, in contrast to ERBIN binding, was not affected by other mutations (Fig. 3C). Mutation of residues in –8 to –14 position in ERBB2 did not affect the interaction with ERBIN or PICK1 (data not shown). We conclude that PICK1 is a new binding partner for ERBB2 that also interacts with additional RTK including ERBB4 and MUSK. From our scanning mutagenesis data, we also demonstrate that ERBIN and PICK1 bind differentially to ERBB2.

Self-interaction between ERBIN and Its PDZ Domain—Oligomerization of PICK1 through its coiled-coil regions has been suggested previously (39). Other PDZ proteins including PSD-95 and EBP-50 oligomerize through disulfide bonds and PDZ-PDZ domain interaction, respectively (40, 41). Using the two-hybrid system, we found that ERBIN (853–1371) containing the ERBIN PDZ domain interacted with itself but not with PICK1 (Fig. 4, A and B). A GST-ERBIN PDZ domain precipitated Myc-tagged ERBIN but not Myc-tagged ERBIN deleted of its PDZ (ERBIN. Δ PDZ). No interaction was found with the DENSIN-180 or AF6 PDZ domain (Fig. 4C). These data suggest that ERBIN is able to oligomerize through a PDZ-PDZ domain interaction.

PICK1 and ERBIN Interact with ERBB2 in Living Cells—To

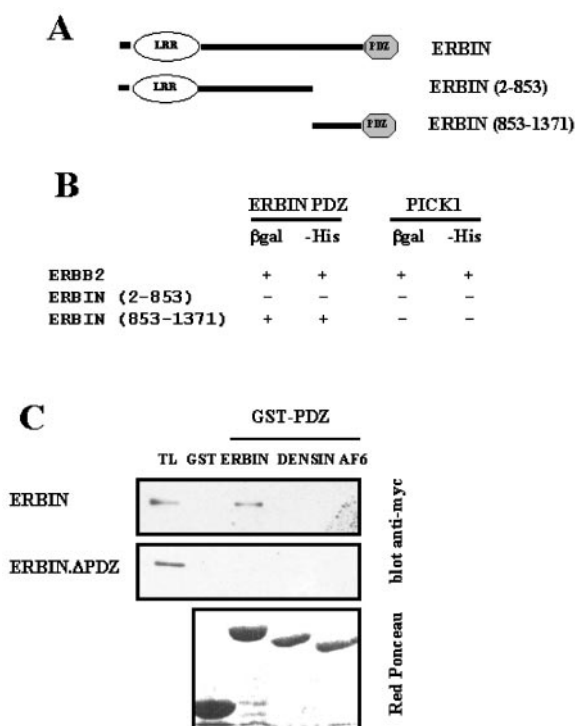


FIG. 4. Self-interaction of the ERBIN PDZ domain. A, schematic representation of full ERBIN (residues 1–1371), ERBIN (2–853) and ERBIN (853–1371). B, LexA-BD-ERBB2 and -ERBIN (2–853) and (853–1371) were transformed in L40 yeast with GAL4-AD-ERBIN PDZ domain or PICK1. Co-transformed yeast were plated on –Trp-Leu-His medium containing 10 mM 3-AT. + means growth on the selective medium (–His) and positive β -galactosidase activity (β gal). C, Myc-tagged ERBIN wild type (ERBIN) or deleted of the PDZ domain (ERBIN. Δ PDZ) expressed in COS cells were pulled down with GST, GST-ERBIN, -DENSIN-180, and -AF6 PDZ domains. Bound proteins were probed with anti-Myc antibody. One tenth of the lysate was run as total lysate (TL). Ponceau Red staining shows comparable amount of GST proteins.

evaluate the interaction of PICK1 with ERBB2 in living cells, we coexpressed a Myc-tagged version of PICK1 with HER1/2, a chimera comprising the extracellular region of EGFR and the intracellular region of ERBB2. Proteins were immunoprecipitated with anti-Myc antibody and resolved by Western blot. Probing of the membrane with anti-ERBB2 antibody revealed that PICK1 interacted with the receptor *in vivo* (Fig. 5A). An identical result was found with Myc-tagged ERBIN. Truncation of the 6 last residues in ERBB2 (HER1/2. Δ 6) abrogated the interaction with PDZ domain proteins (Fig. 5A). ERBIN was a substrate for ERBB2 kinase activity and was unable to coimmunoprecipitate with phosphorylated ERBB2 (Fig. 5B) (6). In contrast, PICK1 efficiently bound to the receptor before and after EGF stimulation. Tyrosine phosphorylation of PICK1 was masked by the immunoglobulin heavy chains (IgH) that comigrated with the protein (Fig. 5B). Tyrosine phosphorylation of ERBIN was dependent on the ERBB2 kinase activity and partially required interaction with the receptor (Fig. 5C). Similarly, we found that PICK1 was phosphorylated by ERBB2 and that deletion of the PDZ domain binding site in ERBB2 reduced this phosphorylation (Fig. 5D). It was recently shown that NHERF, a PDZ protein, binds to PDGF receptors and enhances their signaling capacity in helping the formation of clusters (42). We found no difference in the pattern of tyrosine-phosphorylated proteins from the cell lysates obtained after HER1/2 and HER1/2. Δ 6 activation. HER1/2. Δ 6 retains all ERBB2 autophosphorylation sites involved in its signaling. Furthermore, both receptors interact equally with SHC and GRB2, two major actors of ERBB2 signal transduction after EGF stimulation

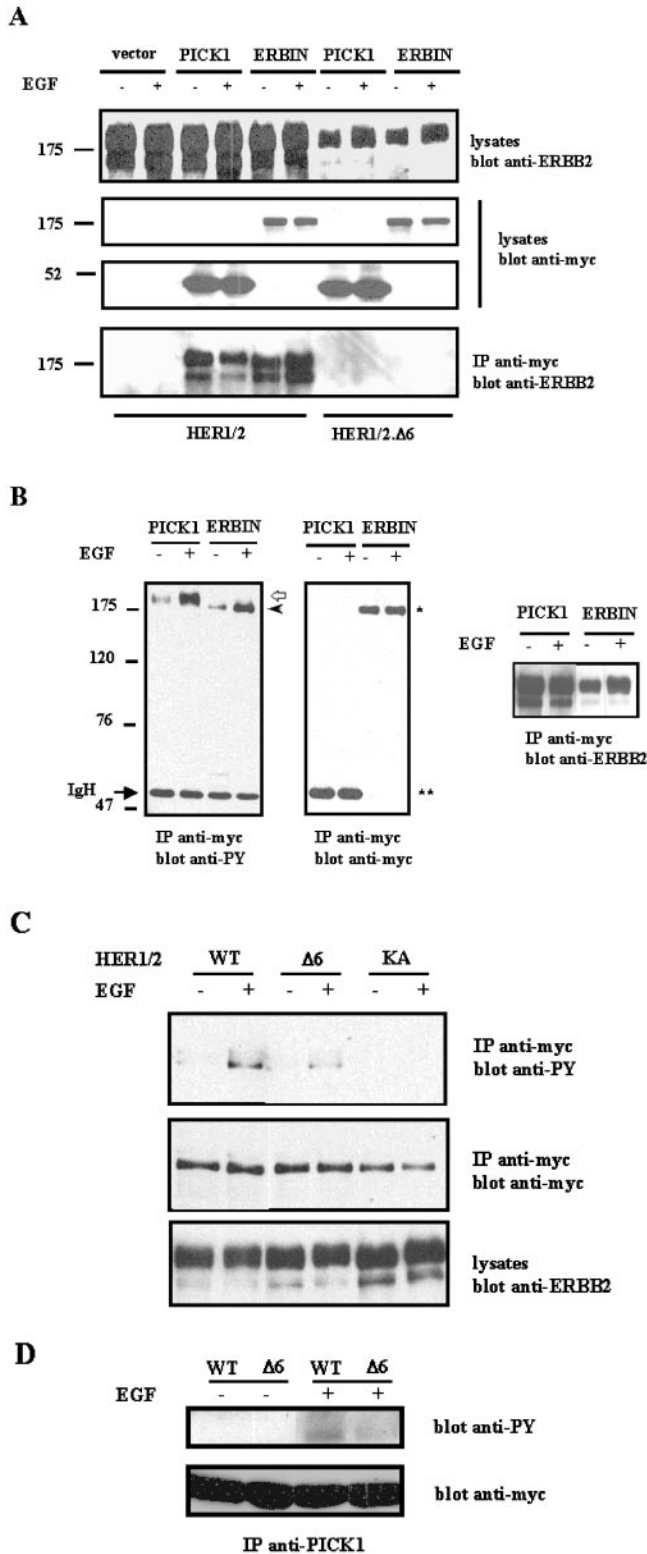


FIG. 5. ERBIN and PICK1 interact with ERBB2/HER2 in cells. A, HER1/2 or HER1/2.Δ6 were coexpressed with Myc-ERBIN or Myc-PICK1 in COS cells treated or not with EGF (100 ng/ml). Proteins were immunoprecipitated with anti-Myc antibody, and bound proteins were revealed with anti-ERBB2 antibody. ERBIN and PICK1 are associated with HER1/2 but not HER1/2.Δ6 (lower panel). Equivalent amount of proteins were evidenced with anti-Myc and anti-ERBB2 antibodies in lysates. B, HER1/2 was transiently coexpressed with Myc-ERBIN and Myc-PICK1 in COS-1 cells. After immunoprecipitation of proteins with anti-Myc antibody, bound proteins were sequentially revealed with anti-PY antibody (left panel) and anti-Myc antibody (middle panel). An empty arrow shows phosphorylated HER1/2 receptor coimmunoprecipitated with PICK1. An arrowhead points to phosphorylated ERBIN.

(data not shown). It is possible that a subset of RTKs, including PDGFR, have a tyrosine kinase activity regulated by PDZ proteins (42) whereas others including ERBB2 and EPHR have not (Refs. 4 and 6; this study).

The PDZ Domain Binding Site in ERBB2 Contains a Retention or Targeting Signal to the Basolateral Membrane of Epithelial Cells—Deletion or mutation of the PDZ domain binding site in ERBB2 provokes a displacement of the receptor from the basolateral to the apical side in epithelial cells (6). This may suggest that the last residues of the receptor act as a targeting or retention signal to the basolateral epithelium. To address more precisely this question, we have grafted the carboxyl terminus of ERBB2 to the well described P75 neurotrophin receptor (P75NTR). P75NTR is a protein mostly found at the apical side of epithelium when ectopically expressed in epithelial cells (43). This localization depends on *O*-glycosylation sites in the ectodomain and on a membrane anchor (Fig. 6A). Deletion of the entire cytoplasmic region of P75NTR does not affect the apical localization of the protein (43, 44). We replaced the 15 last residues of P75NTR with the 15 last residues of ERBB2 (P75-ERBB2) or GBT-1 (P75-GBT1) (Fig. 6A). GBT-1 is a γ -aminobutyric acid transporter retained to the basolateral epithelium through a PDZ domain interaction (45). Populations of MDCK cells stably expressing P75NTR and P75 chimeras were selected, and confocal analysis of the subcellular distribution of ectopic receptors was done at least 3 days after confluence. Double-staining with an antibody directed against Gp114 (in red), a major apical protein in MDCK cells, and ME 20-4 (in green), a monoclonal antibody directed against human P75NTR, was done (22). *Z* sections showed that P75NTR colocalized with the apical marker (see the overlap of red and green in left panel of Fig. 6B), whereas P75-ERBB2 or P75-GBT1 (green) localized to the basolateral membrane and were excluded from the apical membrane in red (Fig. 6B, middle and right panels). Cell surface biotinylation was done on the apical or the basolateral membranes of the same MDCK populations, and the surface expression of the receptors was determined. Although P75NTR was found mainly located to the apical membrane, most of the P75-ERBB2 chimera was basolateral (Fig. 6C). Quantitative analysis of the polarity of P75NTR and the P75-ERBB2 chimera was done after densitometry scanning. P75NTR was enriched on the apical membrane (79.5% apical and 20.5% basolateral) as expected (43), whereas the chimera was almost exclusively found in the basolateral membrane (1% apical and 99% basolateral) (data not shown). We conclude that the ERBB2 PDZ domain binding site acts as a dominant basolateral localization signal in polarized epithelial cells.

DISCUSSION

ERBB2/HER2 is a member of the ERBB RTK subfamily important in the earlier steps of mammalian development and a novel target in breast cancer therapy (46). We aim to identify ERBB2 binding partners and characterize protein complexes im-

Asterisks point to Myc-ERBIN (*) and Myc-PICK1 (**). PICK1 is a 50-kDa protein (see A) that co-migrates with the heavy chains of immunoglobulins (IgH). In the left panel (blot anti-PY), PICK1 is masked by an unspecific signal that labels the IgH. In the right panel, coimmunoprecipitated receptors were revealed with anti-ERBB2 antibody. C, HER1/2 wild type and mutants were transiently expressed in COS-1 cells with Myc-ERBIN. After lysis, anti-Myc antibody was used for immunoprecipitation. After Western blot, the membrane was successively probed with anti-PY and anti-Myc antibodies, respectively. Lysates were probed with anti-ERBB2 in the bottom panel. D, same as C. EGF-treated cells expressing HER1/2 (WT) or HER1/2.Δ6 mutant (Δ6) with Myc-PICK1 were lysed and immunoprecipitated with rabbit anti-PICK1 antibody. Proteins were probed with anti-PY and anti-Myc antibodies, respectively. Tyrosine phosphorylation of PICK1 is decreased when the PICK1/ERBB2 interaction is abrogated.

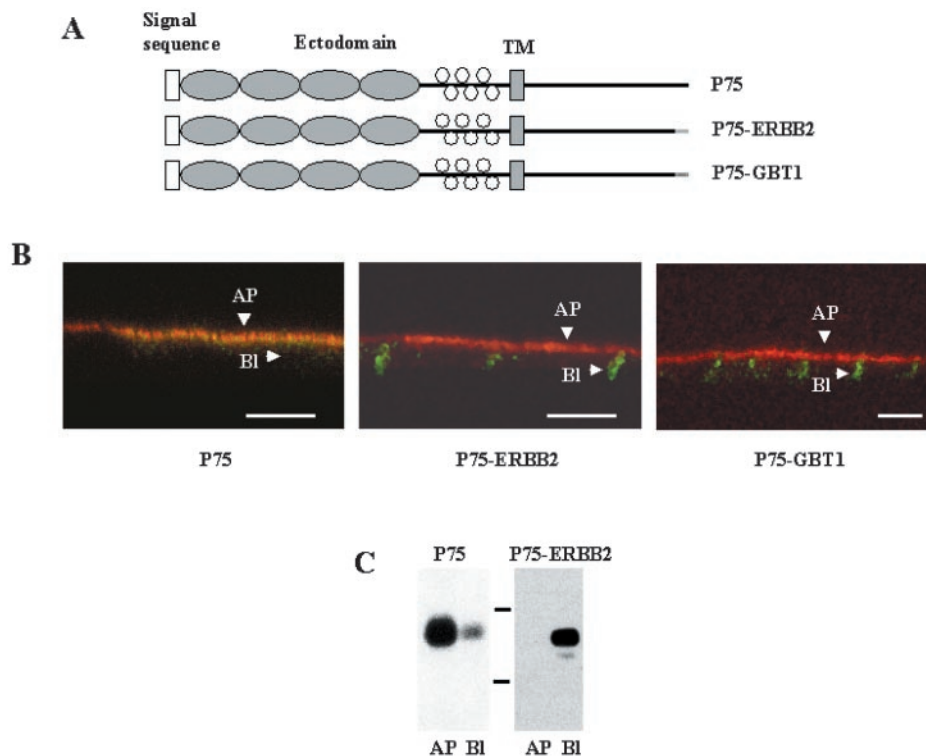


FIG. 6. Localization of P75 chimeras in MDCK cells. *A*, schematic of P75NTR (P75) and P75NTR chimeras where the 15 last residues of P75 are replaced with the 15 last residues of ERBB2 (P75-ERBB2) or GBT-1 (P75-GBT1). *B*, P75NTR (P75) or P75 chimeras were localized by indirect immunofluorescence in MDCK cells and confocal microscopy using Z sections. Gp114, an apical marker of MDCK cells, is labeled in red, and P75NTR or chimeras are labeled in green. Both chimeras are exclusively present at the basolateral membrane (Bl), whereas p75NTR is found mainly at the apical side (AP). Bar is 10 μ m. *C*, transfected MDCK II cells grown on filters and expressing either P75NTR (P75) or P75-ERBB2 were biotinylated from the apical (AP) or the basolateral (Bl) side of the cells. After immunoprecipitation with ME 20-4, proteins were Western-blotted and biotinylated P75NTR or chimeras were revealed using streptavidin coupled to peroxidase. Bars are for markers of molecular masses (from top to bottom, 84 and 62 kDa). Quantitative analysis of the polarity of P75NTR and the P75-ERBB2 chimera was also done after densitometry scanning of selective biotinylation experiments ($n = 2$). P75NTR is enriched at the apical membrane (79.5% apical and 20.5% basolateral), whereas the chimera is almost exclusively found at the basolateral membrane (1% apical and 99% basolateral) (data not shown).

portant for its functions. Beside proteins involved in ERBB2 signaling such as GRB2 and SHC, ERBIN, a PDZ adaptor molecule we recently identified, is most probably required for ERBB2 targeting or retention in polarized cells (6). This paper further describes the interaction between the ERBIN PDZ domain and ERBB2. We have precisely defined the consensus binding site for the ERBIN PDZ domain and identified a panel of receptors containing such motif. Among them are glutamate receptor subunits, *i.e.* NR2B and NR2C, and Kv1.4 channel, which are well known binding partners for PSD-95 in the brain. We did not detect an interaction between the DENSIN-180 PDZ domain (71% identical to ERBIN PDZ domain) and these peptides (data not shown), suggesting that ERBIN and DENSIN-180 bind to different receptors. Interestingly, ERBIN is highly expressed in the brain and shows a post-synaptic distribution in cultured neurons like NMDA receptors.² It is possible that ERBIN participates to the recently characterized multimolecular complex associated to NMDA receptors (47). Future studies will address whether ERBIN interacts with ligands containing a COOH-terminal Ψ XX Ψ [ED][STV]XV motif *in vivo*.

Among LAP proteins, *Drosophila* SCRIBBLE contains four class I PDZ domains in carboxyl terminus and the nematode LET-413 has a single class II PDZ domain. These protein interaction domains have no known target. Obviously, an important issue will be to determine cellular ligands for these orphan PDZ domains. Human SCRIBBLE binds to a SRTRRETQL peptide found in the E6 oncoprotein of human papillomavirus that conforms to a class I PDZ domain binding site

(48). Both human SCRIBBLE and ERBIN are located at the lateral side of epithelial cells where they may share common binding partners (6, 48).

PICK1 is described here as a second PDZ protein binding partner for the ERBB2 receptor. Using different approaches, we show that PICK1 and ERBIN do not interact with the receptor in a similar fashion. First, whereas the ERBIN PDZ domain is sufficient for this interaction, the entire peptide sequence is required in the case of PICK1. The PICK1 PDZ domain is nonetheless indispensable for binding to ERBB2. Second, some mutations in ERBB2 that abrogate the interaction with ERBIN, *i.e.* mutation of residues in -3-, -4-, and -7-positions, do not affect the binding to PICK1. The COOH-terminal VXV motif in ERBB2 is apparently solely required for PICK1. Accordingly, other PICK1 ligands such as MUSK and EPHB2 contain such a motif. The residue in -2-position can be replaced by a serine or a threonine as in the case of ERBB4 and PKC α . Third, PICK1, but not ERBIN, interacts with ERBB2 after tyrosine phosphorylation of the receptor. Interestingly, the binding of PICK1 and GRIP to GluR2 subunit is differentially regulated by serine phosphorylation. GluR2 contains a carboxyl-terminal IESVKI sequence phosphorylated on serine by PKC. PICK1, but not GRIP1, still binds to phosphorylated GluR2 (49). The tyrosine residue in ERBB2 carboxyl-terminal sequence (residue in -7-position) lies very close to the VXV motif of ERBB2 and is a major site of phosphorylation (Fig. 1A). This suggests that phosphorylation of Tyr-1248 abrogates ERBIN, but not PICK1, interaction (6). Post-translational modifications may thus modulate binding of certain PDZ domain

² B. Dargent, personal communication.

proteins to receptors and lead to more dynamic and regulated interactions with phosphatase or kinase activities.

We found that PICK1 is mainly present in the cytosol, the perinuclear region, and the nucleus in epithelial cells (data not shown). Similar findings were published by other groups (39). In contrast, ERBIN is almost exclusively found at the plasma membrane in epithelia (6). PICK1 and ERBIN may thus be involved in different ERBB2 functions in epithelia and participate to different protein complexes. PICK1 and ERBIN can potentially oligomerize and increase the possibilities of interaction. A next step will be to unravel the identity of binding partners for these adaptor proteins and to understand their role in regard to ERBB2 function in normal and pathological situations.

A large number of ligands for PICK1 have been described recently, suggesting that this adaptor may be important for the function of different subsets of receptors. For example, PICK1 clusters EPH receptors and water channel aquaporins in the inner ear, creating a functional complex important for aqueous homeostasis (38). Considering the interaction between PICK1 and RTKs, it is interesting to note that ERBB2 is found at neuromuscular junctions with MUSK and ERBB4, two other PICK1 partners important for neuromuscular transmission (5). Taken together, it appears that PICK1 may gather different receptors at neuromuscular junctions and create a functional scaffold important for neuromuscular physiology. The neuromuscular junction is a well organized structure where growth factors like neuregulins and agrin secreted by the synapse stimulate receptors located at the post-synapse (50). We speculate that PICK1 participates to the machinery involved in the clustering, retention, or targeting of these receptors in this polarized area.

The role of ERBIN and PICK1 in ERBB2 signal transduction remains unclear in contrast to NHERF, a PDZ protein that enhances PDGFR signaling (42). However, the role of PDZ domains in ERBB2 positioning is clearer. We show here that the ERBB2 peptide sequence involved in PDZ domain interaction is sufficient to locate P75NTR, an apical protein, to the basolateral side of epithelial cells. This corroborates our previous finding that deletion of this peptide delocalizes basolateral ERBB2 to the apical membrane in MDCK cells (6). We thus favor the hypothesis that ERBIN and PICK1 are involved in the localization of ERBB2 at specific sites in polarized cells. PDZ proteins participate to protein complexes associated with receptors with tyrosine kinase activity. As binding partners, they are certainly involved in many aspects of RTK physiological functions that have yet to be evaluated.

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**The ERBB2/HER2 Receptor Differentially Interacts with ERBIN and PICK1
PSD-95/DLG/ZO-1 Domain Proteins**

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