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The Erbin PDZ Domain Binds with High Affinity and Specificity to the Carboxyl Termini of δ-Catenin and ARVCF*

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Erbin is a recently described member of the LAP (leucine-rich repeat and PDZ domain) protein family. We used a C-terminally displayed phage peptide library to identify optimal ligands for the Erbin PDZ domain. Phage-selected peptides were type 1 PDZ ligands that bound with high affinity and specificity to the Erbin PDZ domain *in vitro*. These peptides most closely resembled the C-terminal PDZ domain-binding motifs of three p120-related catenins: δ-catenin, ARVCF, and p0071 (DSWV-COOH). Analysis of the interactions of the Erbin PDZ domain with synthetic peptides matching the C termini of ARVCF or δ-catenin also demonstrated specific high affinity binding. We characterized the interactions between the Erbin PDZ domain and both ARVCF and δ-catenin *in vitro* and *in vivo*. The Erbin PDZ domain co-localized and coprecipitated with ARVCF or δ-catenin complexed with β-catenin and E/N-cadherin. Mutagenesis and peptide competition experiments showed that the association of Erbin with the cadherin-catenin complex was mediated by the interaction of its PDZ domain with the C-terminal PDZ domain-binding motifs (DSWV-COOH) of ARVCF and δ-catenin. Finally, we showed that endogenous δ-catenin and Erbin co-localized in and co-immunoprecipitated from neurons. These results suggest that δ-catenin and ARVCF may function to mediate the association of Erbin with the junctional cadherin-catenin complex. They also demonstrate that C-terminal phage-display technology can be used to predict physiologically relevant ligands for PDZ domains.

PDZ[1] domains are 80–100-amino acid compact globular motifs that are usually embedded in larger multidomain scaffolding proteins (1–3). PDZ domains predominantly mediate protein/protein interactions by recognizing the C termini of various intracellular and cell-surface proteins. Type 1 PDZ domains interact with the C-terminal consensus sequence *X*(S/T)*X*(V/I/L)-COOH, whereas type 2 domains bind to the C-terminal consensus sequence *X*-hydrophobe-X-hydrophobe-COOH (3–5). Structural analyses of peptides bound to PDZ domains suggest necessary interactions at both positions 0 and −2 (6–8). However, among type 1 PDZ ligands, these residues are relatively invariant, indicating that other residues within the C terminus likely contribute to the specificity of PDZ domain/ligand interactions (3). For example, two previous studies (9–11), as well as those presented here, demonstrated the importance of residues −1 and −3 for binding specificity and affinity for some PDZ domain/ligand interactions.

Genetic evidence supports a role for several families of PDZ domain-containing proteins as scaffolding molecules that target signaling complexes to various subcellular locations. For example, the multi-PDZ domain protein INAD was found to assemble components of the *Drosophila* visual transduction system to allow for efficient signaling in response to light (12, 13). Another study in the nematode *Caenorhabditis elegans* demonstrated that the basolateral localization of the LET-23 receptor tyrosine kinase is dependent upon direct binding to the PDZ domain of LIN7 (14, 15). Mutations that disrupt this interaction cause the mislocalization of the LET-23 receptor tyrosine kinase and a subsequent loss of LET-23 signal transduction activity. Together with a variety of other studies, these data suggest that a major function of the PDZ domains embedded within scaffolding proteins is to assemble signaling complexes at specific subcellular locations.

Erbin is a recently described member of the LAP protein family (16). LET-413, the ortholog most closely related to human Erbin (and human Densin-180) in the *C. elegans* genome, is a basolateral protein that plays a critical role in the polarization of adherens junctions. Specifically, loss of LET-413 expression causes a failure in apically directed compaction of the adherens junction during epidermal cell maturation and results in defective cell/cell adhesion (17, 18). HER2 is an epidermal growth factor receptor-related tyrosine kinase that is amplified in a high percentage of metastatic breast tumors and is a causal factor in the development of some breast cancers (19). HER2 is found on the basolateral membrane of epithelial cells and has a putative PDZ domain-binding motif at its C terminus that is required for this localization (16). Analogous to the LET-23 receptor tyrosine kinase described above, deletion of the HER2 PDZ domain-binding motif results in the aberrant accumulation of HER2 on the apical plasma membrane, presumably due to an inability to interact with a PDZ domain-containing protein necessary for its targeting to or retention at

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[1] The abbreviations used are: PDZ, PSD-95/Discs Large/ZO-1; Erbin, Erbb2-interacting protein; LAP, leucine-rich repeat and PDZ domain; MAGI, membrane-associated guanylate kinase with inverted orientation; ARVCF, Armadillo protein deleted in velo-cardiofacial syndrome; GST, glutathione *S*-transferase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; RFP, red fluorescent protein; HEK, human embryonic kidney.

the basolateral membrane (16). Erbin has been reported as the protein that mediates basolateral localization of the HER2 receptor through direct binding of its single PDZ domain to the HER2 C terminus (16).

Because of the potential therapeutic value of disrupting HER2 basolateral targeting, we initially set out to identify peptide ligands for the Erbin PDZ domain that could block its interaction with the HER2 C terminus. To do this, we used a recently described phage system that displays a highly diverse and random assortment of short peptides fused to the C terminus of the M13 gene-8 major coat protein (9). Using two PDZ domains from the protein MAGI-3, it was previously demonstrated that this system could be utilized to obtain high affinity peptides that resemble either type 1 or 2 PDZ domain-binding motifs, depending on the PDZ domain used for selection. We used this system to isolate peptides that bind optimally to the Erbin PDZ domain. Surprisingly, analysis of the Erbin PDZ domain-binding peptides revealed that they more closely resembled the C termini of three p120-like catenins (δ-catenin, ARVCF, and p0071) than the C terminus of HER2 (20). Subsequent biochemical and cell biological studies focusing on δ-catenin and ARVCF demonstrated that the interaction with Erbin is of high affinity and specificity. Together, these results suggest that δ-catenin and ARVCF bind to the Erbin PDZ domain *in vivo*.

EXPERIMENTAL PROCEDURES

Isolation of Erbin PDZ Domain-binding Peptides

A previously described library of random heptapeptides fused to the C terminus of the M13 gene-8 major coat protein was cycled through rounds of binding selections with a bacterially expressed GST-Erbin PDZ domain fusion protein coated on 96-well Maxisorp immunoplates (NUNC) as the capture target. Phage were propagated in *Escherichia coli* XL1-Blue (Stratagene) in medium supplemented with M13-VCS helper phage (Stratagene) to facilitate phage production and 10 μM isopropyl-1-thio-β-D-galactopyranoside to induce expression of the library. After three rounds of selection, individual phage were isolated and analyzed in a phage ELISA (21). Phage that bound to the GST-Erbin PDZ domain fusion protein, but not to a control GST fusion protein, were subjected to DNA sequence analysis.

Binding Assays

The binding affinities of peptides for the Erbin PDZ domain were determined as IC_{50} values using a previously described competition ELISA (9). The IC_{50} value was defined as the concentration of peptide that blocked 50% of PDZ domain binding to immobilized peptide. Assay plates were prepared by immobilizing an N-terminally biotinylated peptide (TGWETWV-COOH) on Maxisorp plates coated with neutravidin (Pierce) and blocked with bovine serum albumin (Sigma). A fixed concentration of the GST-Erbin PDZ domain fusion protein (50 nM) in PBT buffer (PBS containing 0.5% bovine serum albumin and 0.1% Tween 20 (Sigma)) was preincubated for 3 h with serial dilutions of peptide and then transferred to the assay plates. After a 15-min incubation, the plates were washed with PBS containing 0.05% Tween 20, incubated with a mixture of anti-GST antibody (0.5 μg/ml; Zymed Laboratories Inc.) and horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (1:2000 dilution; Jackson ImmunoResearch Laboratories, Inc.) in PBT buffer, washed again, and detected with 3,3′,5,5′-tetramethylbenzidine/H_2O_2 peroxidase substrate (Kirkegaard & Perry Laboratories Inc.)

Data Base Search of Protein C Termini

A motif-searching algorithm was used to identify proteins with C termini that resemble the phage-displayed peptides selected for binding to the Erbin PDZ domain. Alignment of phage-derived peptides established a clear consensus sequence of (D/E)(T/S)WV-COOH as the preferred motif for tight binding to the Erbin PDZ domain. We used this consensus sequence to search the Dayhoff Database, restricting our search criteria to the C-terminal residues of proteins within the data base. Using these criteria, we identified 25 proteins that end with this C-terminal motif. Non-vertebrate proteins as well as one extracellular protein (PDZ domains are cytoplasmic) were manually filtered out, leaving a total of 18 sequences that fit our criteria. Of these, several are orthologs or simply separate GenBank™/EBI Data Bank entries of the same gene product. Final examination of the 18 sequences indicated that three unique intracellular gene products were represented: δ-catenin, ARVCF, and p0071 (plakophilin-4). These three proteins are all members of the Armadillo family of proteins, which, based on their four C-terminal residues, are candidate *in vivo* ligands for the Erbin PDZ domain.

Plasmids and Constructs

Mammalian—Expression constructs containing the six individual PDZ domains of MAGI-3 were constructed by PCR cloning using a full-length cDNA of human MAGI-3 (22) cloned into the pcDNA3.1V5/His-TOPO cloning vector (Invitrogen) as the template. The PDZ1 (amino acids 417–535), PDZ2 (amino acids 584–707), PDZ3 (amino acids 741–840), and PDZ4 (amino acids 870–976) domains were cloned into the *Bam*HI/*Not*I sites of pEBG, creating fusions with the C terminus of GST. Regions of MAGI-3 containing the PDZ0 (amino acids 1–406) and PDZ5 (amino acids 980–1150) domains were cloned into the *Hin*dIII/*Sal*I sites of pEGFP-N3 (CLONTECH), creating fusions with the N terminus of EGFP. The PDZ domain of human Erbin (amino acids 1273–1371, corresponding to GenBank™/EBI Data Bank accession number AF263744) was amplified from expressed sequence tag AA992250 and cloned into three different vectors: 1) the pcDNA3.1NT/GFP-TOPO TA cloning vector (Invitrogen), creating a fusion with the C terminus of GFP; 2) the *Xho*I/*Sac*II sites of pDsRed1-N1 (CLONTECH), creating a fusion with the N terminus of RFP; and 3) the *Bam*HI/*Not*I sites of pEBG, creating a fusion with the C terminus of GST. Human HER2 was cloned into pRK, and this construct has been previously described (23). Human δ-catenin and δ-catenin Δc6, a form of δ-catenin missing the C-terminal six amino acid residues, were PCR-cloned into pEGFP-C1 (CLONTECH), creating fusions with the C terminus of EGFP. A proteolytically stable mutant form of β-catenin (24) was cloned into pEGFP-C1, creating a fusion with the C terminus of EGFP. Human ARVCF cloned into the pcDNA3.1 vector (Invitrogen) has been described (25) and was a gift from the laboratory of Dr. Albert Reynolds. Full-length Myc-tagged Erbin has been described (26) and was a gift from the laboratory of Dr. Lin Mei.

Prokaryotic—The Erbin PDZ domain (amino acids 1217–1371) or the MAGI-3 PDZ2 domain (amino acids 584–707) were cloned into the *Eco*RI/*Not*I or *Bam*HI/*Not*I sites of the *E. coli* expression vectors pGEX6P-1 and pGEX4T-3 (Amersham Biosciences), respectively. The PDZ domain of human Densin-180 (amino acids 1424–1537, corresponding to GenBank™/EBI Data Bank accession number AF434715) was cloned into the *Bam*HI/*Not*I sites of pGEX. Expression and affinity purification of *E. coli* cell-expressed GST proteins (Amersham Biosciences) were carried out as recommended by the manufacturer.

Protein Extracts

HEK 293 and Caco-2 cells were grown in high glucose Dulbecco's modified Eagle's medium, 10% fetal calf serum, 1× nonessential amino acid supplement, 1× L-glutamine supplement, 10 mM HEPES (pH 7.4), and penicillin/streptomycin (Invitrogen). For heterologous expression of proteins, HEK 293 cells were grown to ~70% confluence and transfected with DNA using FuGENE 6 reagent (Roche Molecular Biochemicals). Transfected cells (20–36 h) were rinsed with PBS, scraped into 1 ml/10-cm dish of homogenization buffer (20 mM Tris (pH 7.5), 1% Triton X-100, 125 mM NaCl, 1 mM dithiothreitol, and protease inhibitor mixture with EDTA (Roche Molecular Biochemicals)), incubated on ice for 10 min, triturated five times, and clarified by centrifugation for 10 min at 12,000 rpm in a refrigerated Eppendorf tabletop centrifuge. Protein concentrations in the clarified extracts were determined using BCA reagents (Pierce) and then normalized for all transfectants by addition of homogenization buffer. Extracts were snap-frozen as aliquots in a dry ice/ethanol bath and stored at −80 °C until used. Caco-2 extracts were prepared from cells grown for 10 days post-confluence. Cells from each 10-cm dish were homogenized with five strokes of a Dounce with a loose pestle (Wheaton) in 1 ml of homogenization buffer, and subsequent extracts were then prepared and stored exactly as described for HEK 293 cells. For brain extracts, mouse brain neocortices were dissected and homogenized in homogenization buffer using 5 ml of buffer/g of tissue. Lysates were precleared at 13,000 × *g* for 20 min at 4 °C. Protein concentrations in the clarified extracts were determined using BCA reagents. Extracts were snap-frozen as aliquots in a dry ice/ethanol bath and stored at −80 °C until used.

Peptide and GST Pull-down Assays and Immunoprecipitations

For peptide pull-down experiments with individually expressed PDZ domains (see Fig. 1), 50–100 μl of HEK 293 cell extract was diluted to

400 μl with homogenization buffer and incubated with 10 μM N-terminally biotinylated peptide and 100 μl of streptavidin-agarose (Sigma) for 2 h on a rotator at 4 °C. The beads were washed three times with 1 ml of binding buffer and boiled in 60 μl of Laemmli reducing sample buffer, 15 μl of which was loaded onto SDS gels along with 10 μl of diluted extract for immunoblot analysis. Peptide pull-down experiments with Caco-2 cell extracts were slightly modified such that 700 μl of undiluted protein extract and 150 μl of streptavidin-agarose beads were used for these experiments.

For GST pull-down experiments with HEK 293 cell-expressed δ-catenin, δ-catenin Δc6, ARVCF, β-catenin, and HER2, 100 μl of extract was diluted to 800 μl in homogenization buffer and re-clarified by centrifugation. The diluted extracts were then incubated with 3 μg of *E. coli* cell-expressed GST fusion protein plus 35 μl of glutathione-Sepharose (Amersham Biosciences) for 2 h on a rotator at 4 °C. Beads were washed three times, and bound proteins were detected by immunoblot analysis. For GST pull-down assays of brain, 1 mg of total brain extract was diluted to a final concentration of 1 mg/ml with homogenization buffer and then re-clarified by centrifugation. Using 800 μl of the diluted clarified extract, GST pull-down assays were carried out exactly as described for HEK 293 cell extracts. For coprecipitation and co-immunoprecipitation experiments (see Fig. 2), cotransfected HEK 293 cells were solubilized in 1 ml of homogenization buffer/10-cm dish, and clarified extracts were obtained as described above. 800 μl of fresh undiluted extract was then mixed with 35 μl of glutathione-Sepharose for coprecipitation or with 5 μg of anti-Myc monoclonal antibody + 30 μl of protein A/G-Sepharose (Pierce) for co-immunoprecipitation and incubated for 2 h at 4 °C on a wheel. The beads were washed three times with 1 ml of homogenization buffer, and precipitated proteins were detected by immunoblotting.

To test whether Erbin PDZ domain-binding peptides could block *in vivo* coprecipitations, cotransfected cells were incubated with peptides for either 6 h (Antennapedia-coupled peptides) or 24 h (fluorescein-coupled peptides) prior to cell harvest. The Antennapedia peptide (CRQIKIWFQNRRMKWKK) was disulfide-linked to a cysteine residue located at the N terminus of either a high affinity phage-derived peptide (CTGWETWV) or a cognate Val-to-Ala substituted negative control peptide (CTGWETWA). Entry into the reducing intracellular environment results in cleavage of the disulfide bond, thus releasing the free PDZ domain-binding peptide in the cytoplasm.

For co-immunoprecipitations from brain, brain extracts were thawed, diluted to 1 mg/ml in homogenization buffer, centrifuged, and pre-absorbed against normal mouse IgG (Santa Cruz Biotechnology) bound to protein A/G-Sepharose for 1 h on a rotating wheel. The pre-clarified brain extracts (0.5 ml) were then immunoprecipitated with 5 μg of the indicated monoclonal antibody and 20 μl of protein A/G-Sepharose. Immunoprecipitated protein-antibody complexes were eluted from the beads by boiling for 5 min in the presence of 30 μl of 2× Laemmli sample buffer. 15 μl of the immunoprecipitated samples and 20 μl of both pre- and post-immunoprecipitation extracts were loaded onto 6% Tris/glycine-acrylamide gels and transferred to nitrocellulose for immunoblot analysis. For peptide-blocking experiments, the only modification was that, prior to immunoprecipitations, clarified extracts were preincubated with peptides for 2 h on ice.

Co-localization in Transfected HEK 293 Cells

HEK 293 cells were grown to 70% confluence on collagen IV-coated coverslips and then transfected with the indicated DNA constructs using FuGENE 6 reagent. 24 h post-transfection, the cells were washed with PBS, fixed for 30 min in 2.5% formaldehyde, permeabilized with 0.25% Triton X-100 in PBS, and blocked with 5% donkey serum. The Erbin PDZ domain and δ-catenin constructs were RFP and EGFP fusions, respectively; and thus, they could be directly visualized. ARVCF and HER2 were visualized by staining cells with antibodies directed against these proteins and with fluorescein-coupled anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.). Coverslips were mounted on slides using Vectashield mounting medium with 4,6-diamidino-2-phenylindole (Vector Labs, Inc.) and sealed with rubber cement. Images were taken on a Zeiss Axiophot fluorescence microscope attached to a digital CCD camera with a ×63 oil immersion objective using SPOT imaging software (Research Diagnostics Inc.).

Immunostaining of Brain Cortex and Cultured Hippocampal Neurons

Hippocampal neurons were dissected from the cortices of embryonic day 18 rats (Taconic Farms Inc.), dissociated, and plated onto poly-L-lysine-coated coverslips in neurobasal medium supplemented with 2 mM glutamine and vitamin B_{27} supplements (Invitrogen). After 3 weeks in culture, neurons were fixed for 15 min in 4% paraformaldehyde in PBS (pH 7.4), washed with PBS, and permeabilized using 0.25% Triton X-100 in PBS. Nonspecific sites were blocked with 10% goat serum diluted in 0.025% Triton X-100 in PBS. Primary antibodies were diluted to a final concentration of 2 μg/ml for Erbin and 2.5 μg/ml for δ-catenin in 1% goat serum and 0.025% Triton X-100 in PBS. The neurons were incubated with primary antibody overnight at 4 °C and then washed three times with PBS before being placed with Alexa 594-conjugated goat anti-rabbit antibody (Molecular Probes, Inc.) diluted to a final concentration of 4 μg/ml in PBS for 1 h at room temperature. The neurons were washed again three times with PBS and incubated with Alexa 484-conjugated goat anti-mouse antibody diluted to a final concentration of 4 μg/ml in PBS for 1 h at room temperature. The coverslips were washed twice with PBS, followed by a 5-min wash with Sörenson's phosphate buffer (pH 7.4) to rinse off excess salt. Finally, the coverslips were mounted on microscope slides using an aqueous mounting solution supplemented with antifade reagents (Gel/Mount, Biomeda). Images were acquired using a Zeiss Axiovert S100 microscope with a ×100 objective coupled to a Bio-Rad MRC1024 confocal laser scanning unit equipped with a krypton/argon lamp. Any pixel labeled with an emitted fluorescent signal of intensity >130 units/pixel in both the red and green channels was considered to be a region of protein co-localization.

Female adult Sprague-Dawley rats (Taconic Farms Inc.) were anesthetized with an intraperitoneal injection of 3% chloral hydrate solution (3.5 mg/10 g of body weight) and transcardially perfused using an ice-cold fix solution composed of 4% paraformaldehyde and 7% picric acid in 0.1 M Sörenson's phosphate buffer (pH 7.4). The cerebellum and cortex were removed and post-fixed for 2 h in the fix solution at 4 °C and then transferred overnight to a solution of 30% sucrose and 0.1 M Sörenson's phosphate buffer at 4 °C. 30-μm-thick coronal sections were cut using a freezing microtome. Free floating sections were rinsed with 0.1 M PBS and blocked with 1% normal goat serum (Vector Labs, Inc.) and 0.02% Triton X-100 in PBS for 1 h at room temperature. The sections were then incubated overnight at 4 °C with primary antibody diluted in 1% normal goat serum and 0.02% Triton X-100 in PBS. Anti-Erbin and anti-δ-catenin antibodies were diluted to 2 and 2.5 μg/ml final concentrations, respectively. Sections were washed three times with PBS and then incubated for 1 h with Alexa 594-conjugated goat anti-rabbit antibody diluted to a 4 μg/ml final concentration in 1% normal goat serum in PBS. Excess antibody was removed by three washes with PBS. The sections were then incubated with Alexa 488-conjugated goat anti-mouse antibody diluted to a final concentration of 4 μg/ml in 1% normal goat serum in PBS. Finally, the sections were washed twice with PBS and once with 0.1 M Sörenson's phosphate buffer before being mounted onto positively charged slides (ProbeOn Plus, Fisher). The sections were imaged as described above.

Antibodies

Anti-Erbin antibodies were obtained from rabbits injected with residues 1217–1371 of this protein fused to GST. Antibodies were affinity-purified against the antigen immobilized on cyanogen bromide-activated Sepharose. From this purified pool of antibodies reactive against both GST and Erbin, the GST-reactive antibodies were completely depleted against similarly immobilized GST-Sepharose, creating pure pools of antibodies against either Erbin or GST. Monoclonal antibodies against δ-catenin, p120 catenin, β-catenin, and γ-catenin were from BD Transduction Labs. Rabbit anti-GFP antibody was from CLONTECH. Rabbit anti-HER2 antibody was from Santa Cruz Biotechnology. Rabbit anti-LIN7-3 antibody (Velis-3) and monoclonal antibodies against N-cadherin or E-cadherin (HECD-1) were from Zymed Laboratories Inc. Anti-ARVCF antibodies were a gracious gift from the laboratory of Dr. Albert Reynolds.

RESULTS

Peptides Selected for Binding to the Erbin PDZ Domain Identify p120-like Catenins as Candidate Protein Ligands—Previously described C-terminally displayed phage peptide libraries (9) were utilized to select peptides that bound with high affinity to a bacterially expressed protein consisting of the PDZ domain of Erbin fused to GST. Phage particles that bound to the Erbin PDZ domain were isolated, and the sequences of the displayed C-terminal peptides were examined. A clear consensus sequence was present in the phage-selected peptides (Table I). As has been found with virtually all previously examined ligands for type 1 PDZ domains (3), the C-terminal residue

TABLE I
PDZ ligands

Sequence[a]	Position[b]						
	−6	−5	−4	−3	−2	−1	0
1	S	G	**W**	**D**	**T**	**W**	**V**
2	T	D	**W**	**E**	**T**	**W**	**V**
3	W	W	**F**	**D**	**T**	**W**	**V**
4	S	G	**W**	**D**	V	**W**	**V**
5				**E**	**T**	**W**	**V**
6	C	S	**L**	**D**	**T**	**W**	**V**
7			**F**	**D**	**S**	**W**	**V**
8		I	S	**E**	**T**	**W**	**V**
9	R	G	**W**	**D**	**T**	**W**	**V**
10				**D**	**T**	**W**	**V**
11	S	E	**F**	**D**	**S**	**W**	**V**
12	S	Q	**W**	**E**	**T**	**W**	**V**
13	T	G	**W**	**E**	**T**	**W**	**V**
14	S	W	**I**	**E**	**T**	**W**	**V**
CONS			Aromatic/hydrophobic	**D**	**T**	**W**	**V**
				E	**S**		
δ-CAT	A	S	P	**D**	**S**	**W**	**V**
ARVCF	Q	P	**V**	**D**	**S**	**W**	**V**
p0071	G	S	P	**D**	**S**	**W**	**V**
HER2	L	G	**L**	**D**	V	P	**V**

[a] Sequences for the following PDZ ligands are listed: phage-displayed ligands selected after three rounds of binding to the Erbin PDZ domain; the consensus sequence for high affinity binding to the Erbin PDZ domain (CONS) derived from sequence similarity among the phage-displayed sequences; the C termini of candidate natural ligands for the Erbin PDZ domain, identified either in this study with data base mining (δ-catenin (δ-CAT), ARVCF, and p0071) or in a previous study with yeast two-hybrid screening (HER2). Sequences that match the consensus sequence (CONS) are shown in boldface.

[b] Positions in the peptide ligand, from the C terminus to the N terminus, are designated 0, −1, etc.

(position 0) was found to be hydrophobic. The Erbin PDZ domain specifically favored Val over Ile and Leu, which are also commonly found at this position. The residue selected at position −1 was exclusively Trp. Position −2 was observed to be either Thr or Ser, with a rare Val residue selected. Because of the preponderance of Thr or Ser at this position, the Erbin PDZ domain appears to be a type 1 domain. A strong consensus for an acidic residue (Glu or Asp) was observed at position −3. A more variable consensus was found at position −4, where many of the selected peptides contained a hydrophobic or aromatic residue. No obvious consensus was detectable at position −5 or −6.

Comparison of the selected consensus sequence ((DE)(T/S)WV-COOH) with the C-terminal sequence of HER2 and all C-terminal sequences in the current Human Genome Database revealed that the Erbin PDZ domain may bind to multiple proteins (Table I). Interestingly, the only human protein C termini that match this consensus sequence belong to a set of three related proteins in the p120 catenin family (which all terminate with the sequence DSWV-COOH) (20). In contrast, examination of the HER2 C terminus revealed some conservation with the phage-selected peptides, but also some significant differences. Val at position −2 is notable because this would be the type of residue preferred by a type 2 PDZ domain, whereas the Erbin PDZ domain, based upon conservation of His^{1347} and phage-selected peptide sequences, appears to be a type 1 PDZ domain. Nonetheless, Val at position −2 was present in a single phage-selected peptide, suggesting that binding of a type 2 ligand is possible, although not preferred. The HER2 C terminus also has Pro at position −1, whereas all phage-selected peptides against the Erbin PDZ domain as well as the C termini of the three p120-like catenins have Trp at this position. Together, these results suggest that the Erbin PDZ domain may bind most optimally to the p120-like catenins δ-catenin, ARVCF, and p0071.

To quantify the interactions between the Erbin PDZ domain and various peptides, we used an ELISA designed to measure the intrinsic binding affinities between the Erbin PDZ domain and peptides free in solution. We (9) and others (3, 27) feel that this type of analysis more accurately reflects the true binding affinities of PDZ ligands because the peptides in solution are monomeric; and thus, the assay is free from artifacts due to avidity effects that can arise with immobilized ligands. Table II illustrates that the affinities of the phage-selected peptides are relatively high, with the sequence TGWETWV-COOH binding at submicromolar levels ($IC_{50} \sim 0.6\ \mu M$). Peptides derived from the C termini of ARVCF (QPVDSWV-COOH) or δ-catenin (ASPDSWV-COOH) bound the Erbin PDZ domain with affinities in the low micromolar range (Table II). Although these affinities are lower than that of the phage-optimized peptide, they are much higher than the affinities measured for the PDZ domain-binding C-terminal motifs of HER2 (EYLGLDVPV-COOH) and β-catenin (AWFDTDL-COOH) (Table II).

It is apparent that deviations from the optimal consensus-binding sequence for the Erbin PDZ domain result in reduced ligand affinity. Even within the allowable residues at each position as defined by the phage display, there is likely a rank order of preference. For example, the substitution of Asp for Glu at position −3 reduced peptide affinity by ~5-fold (compare TGWDTWV with TGWETWV in Table II), even though Glu and Asp are both well represented among the phage selectants (Table I). Larger deviations from the consensus sequence resulted in correspondingly larger reductions in affinity, as evidenced by the effects of substitutions at position −1, where replacement of the highly conserved Trp with either Phe (the most similar natural amino acid) or Ala led to very large decreases in affinity (Table II). Thus, ARVCF and δ-catenin bind the Erbin PDZ domain with affinities close to those of optimal phage-derived ligands because their C-terminal sequences almost perfectly match the phage-derived consensus-binding motif. In contrast, both HER2 and β-catenin exhibit significant mismatches when their C termini are compared with the high affinity consensus sequence (especially at position −1); and consequently, the C termini of these proteins exhibit extremely low affinities for the Erbin PDZ domain. Taken together, these results suggest that, based solely on their C-terminal sequences, ARVCF and δ-catenin (and likely p0071) are high affinity ligands for the Erbin PDZ domain.

TABLE II
IC_{50} values for PDZ domain-binding synthetic peptides

The IC_{50} values are the concentrations of peptide that blocked 50% of PDZ domain binding to immobilized peptide in an ELISA. Peptides were synthesized with acylated N termini.

Source[a]	Position									IC_{50}
	−8	−7	−6	−5	−4	−3	−2	−1	0	
										μM
Phage[b]			T	G	W	E	T	W	V	0.6
Phage[b]			T	G	W	D	T	W	V	2.8
Phage[c]					W	E	T	F	V	180
Phage[c]					W	E	T	A	V	560
Phage[d]			A	T	Q	I	T	W	V	480
ARVCF			Q	P	V	D	S	W	V	14
δ-Catenin			A	S	P	D	S	W	V	37
β-Catenin			A	W	F	D	T	D	L	2100
HER2	E	Y	L	G	L	D	V	P	V	2200

[a] The synthetic peptides correspond either to sequences selected from peptide phage libraries or to the C termini of natural human proteins, as indicated.
[b] Selected for binding to the Erbin PDZ domain.
[c] Designed to test the effect of altering Trp at position −1 of high affinity peptides selected for binding to the Erbin PDZ domain.
[d] Selected for binding to the MAGI-3 PDZ2 domain (9).

δ-Catenin and ARVCF Bind Strongly and Specifically to the Erbin PDZ Domain—To examine whether a short C-terminal peptide is sufficient to interact with the Erbin PDZ domain, we investigated the ability of these fragments to precipitate fusion proteins containing the PDZ motif. Previously, we used the second PDZ domain of MAGI-3 as a target for C-terminal phage display and obtained the high affinity ligand ATQITWV-COOH (9, 22). We examined the specificity of this peptide as well as that of the Erbin-binding peptide (TGWETWV-COOH) in co-precipitation experiments using all six PDZ domains from the MAGI-3 protein (22) as well as the Erbin PDZ domain. Fig. 1*A* illustrates that each biotinylated peptide interacted specifically and exclusively with the PDZ domain against which it was selected. Thus, the ATQITWV-COOH peptide precipitated only the MAGI-3 PDZ2 domain, whereas the TGWETWV-COOH peptide precipitated only the Erbin PDZ domain. We noted that the two phage-selected peptides were identical at three of the four most critical residues for PDZ binding; and yet, in both the ELISA (Table II) and the pull-down assay, the peptide selected for binding to the MAGI-3 PDZ2 domain bound poorly to the Erbin PDZ domain. Therefore, we examined whether the glutamate residue at position −3 of the Erbin PDZ domain-selected peptide would confer Erbin PDZ domain-binding specificity on the MAGI-3 PDZ2 domain-selected peptide. We synthesized a peptide (ATQETWV-COOH) by substituting Ile at position −3 of the MAGI-3 PDZ2 domain-selected peptide for Glu found in the Erbin PDZ domain-selected peptide. Fig. 1*A* shows that the new peptide interacted well with the Erbin PDZ domain, but not with the MAGI-3 PDZ2 domain, demonstrating the importance of residue −3 in binding discrimination. These results are also consistent with analysis of phage-selected peptides against the Erbin PDZ domain, which showed that the last four residues are most invariant. However, the phage selections also suggested some preference for a hydrophobic amino acid at position −4 in Erbin PDZ domain-binding peptides. Clearly, the contribution of the side chain at position −4 is small relative to the combined contributions of the side chains at positions 0 through −3, and this contribution did not register in the *in vitro* pull-down assay. Finally, we used these peptides to show that the Ile-to-Glu change is sufficient for discriminate precipitation of endogenous Erbin, but not the single PDZ domain-containing protein LIN7-3 (Velis-3), from Caco-2 cell lysates (Fig. 1*B*).

Pull-down experiments using a bacterially produced GST fusion of the Erbin PDZ domain were performed to determine whether full-length ARVCF and δ-catenin are in fact high affinity ligands for the Erbin PDZ domain, as suggested by the peptide binding studies. Fig. 1*C* illustrates that δ-catenin and ARVCF were efficiently precipitated when incubated with the GST-Erbin PDZ domain fusion. The role of the C terminus in the δ-catenin interaction was demonstrated by the observation that a mutant form of δ-catenin lacking the C-terminal six amino acids was unable to bind to the Erbin PDZ domain. Fig. 1*C* also illustrates that neither HER2 nor β-catenin was efficiently precipitated from transfected cell extracts by the GST-Erbin PDZ domain fusion protein, although we were able to detect a small amount of HER2 in the precipitate. These data suggest that, as predicted from the peptide affinity measurements in Table II, δ-catenin and ARVCF bind much more tightly to the Erbin PDZ domain than do HER2 and β-catenin.

An interaction between HER2 and the Erbin PDZ domain has been previously reported (16), and the slight HER2 precipitate observed in Fig. 1*C* suggested that this interaction resulted from the binding of the HER2 C terminus to the peptide-binding site of the Erbin PDZ domain. Thus, we used competition assays to determine whether δ-catenin and HER2 bind to the same site on the Erbin PDZ domain as do the phage-selected peptides. Fig. 1*D* illustrates that a phage-selected peptide was able to effectively compete with the interactions between the Erbin PDZ domain and δ-catenin or HER2, suggesting that both proteins interact with the peptide-binding site of the Erbin PDZ domain. As expected from the affinity measurements, the interaction between δ-catenin and the Erbin PDZ domain appears to be stronger than that between HER2 and the Erbin PDZ domain. Interestingly, the peptide (ATQITWV-COOH) that bound to the MAGI-3 PDZ2 domain and that shares three residues with the Erbin PDZ domain-binding peptide (TGWETWV-COOH) was completely ineffective at competing the δ-catenin interaction, but it showed a significant level of competition with the HER2 interaction, suggesting that other low affinity (see Table II) interactions may also occur with the peptide-binding site of the Erbin PDZ domain.

δ-Catenin and ARVCF Interact with Erbin in Transfected Cells—Membrane localization of p120-like catenins is reportedly mediated by an interaction between their Armadillo repeats and the juxtamembrane region of cadherin adhesion proteins (28). Previous reports demonstrated that δ-catenin associates with the cell surface at points of cell/cell contact when expressed heterologously (29). In contrast, the Erbin PDZ domain has a diffuse cytoplasmic localization when expressed alone in HEK 293 cells (Fig. 2*A*). Fig. 2*B* illustrates that, when coexpressed, the Erbin PDZ domain and δ-catenin completely co-localized, resulting from the recruitment of the Erbin PDZ

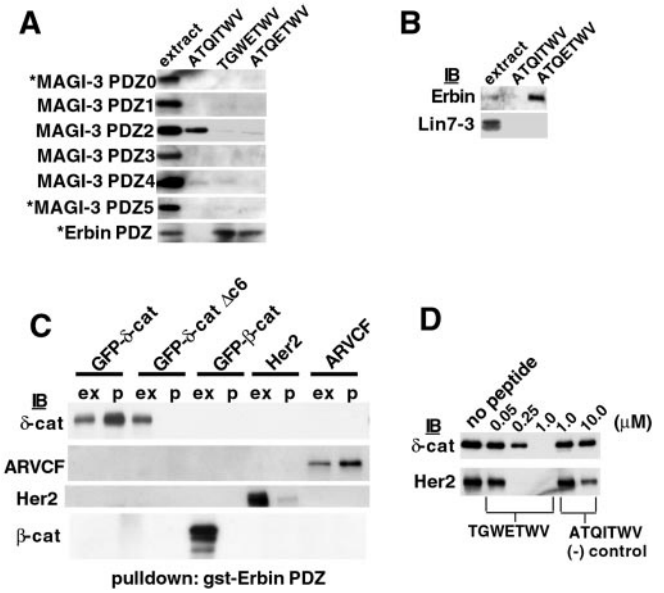

FIG. 1. **Phage-selected peptides bind specifically to the Erbin PDZ domain *in vitro*, as do protein ligands predicted from their consensus sequences.** *A*, the Erbin PDZ domain and six control PDZ domains were expressed in HEK 293 cells as GFP (indicated by *asterisks*) or GST fusions, and extracts were made from each. Biotinylated versions of peptides phage-selected for high affinity binding to either the MAGI-3 PDZ2 domain (ATQITWV-COOH) or the Erbin PDZ domain (TGWETWV-COOH) were tested for their ability to precipitate the various PDZ domains. Each peptide specifically precipitated only the PDZ domain against which it was selected. A single substitution of Glu for Ile at position −3 of the MAGI-3 PDZ2 domain-specific peptide produced a peptide (ATQETWV-COOH) that no longer precipitated the MAGI-3 PDZ2 domain, but specifically precipitated only the Erbin PDZ domain (*right lane*). Peptide concentrations were 10 μM, and readout was by immunoblotting with either anti-GFP or anti-GST antibodies. *B*, the indicated peptides (10 μM) were assayed for their ability to precipitate Erbin from Caco-2 cell extracts. Immunoblot (*IB*) analysis with the indicated antibodies showed that Erbin was precipitated with a peptide ending in ETWV, but not ITWV. Similar to Erbin, LIN7-3 has a single PDZ domain, but was not precipitated by either peptide. *C*, potential protein ligands for the Erbin PDZ domain were expressed in HEK 293 cells and assayed for their ability to be precipitated by 3 μg of *E. coli* cell-expressed GST-Erbin PDZ domain fusion protein. The expressed proteins were as follows: ARVCF and δ-catenin (*δ-cat*), the previously reported putative Erbin PDZ ligand HER2, and the negative controls δ-catenin Δc6 and β-catenin. For immunoblot analysis with the indicated antibodies, 1% of the precipitated extracts or 30% of the precipitated protein was used. *D*, precipitation of δ-catenin or HER2 by 6 μg of *E. coli* cell-expressed GST-Erbin PDZ domain fusion protein could be efficiently blocked by co-incubation with a phage-selected peptide (TGWETWV-COOH) that binds the Erbin PDZ domain with high affinity, but not by a control peptide (ATQITWV-COOH). Note that 1 μM peptide was approximately a 3-fold molar excess over the Erbin PDZ domain. Immunoblot analysis showed the relative amounts of δ-catenin or HER2 precipitated by the GST-Erbin PDZ domain fusion protein in the absence or presence of varying concentrations of the indicated peptide.

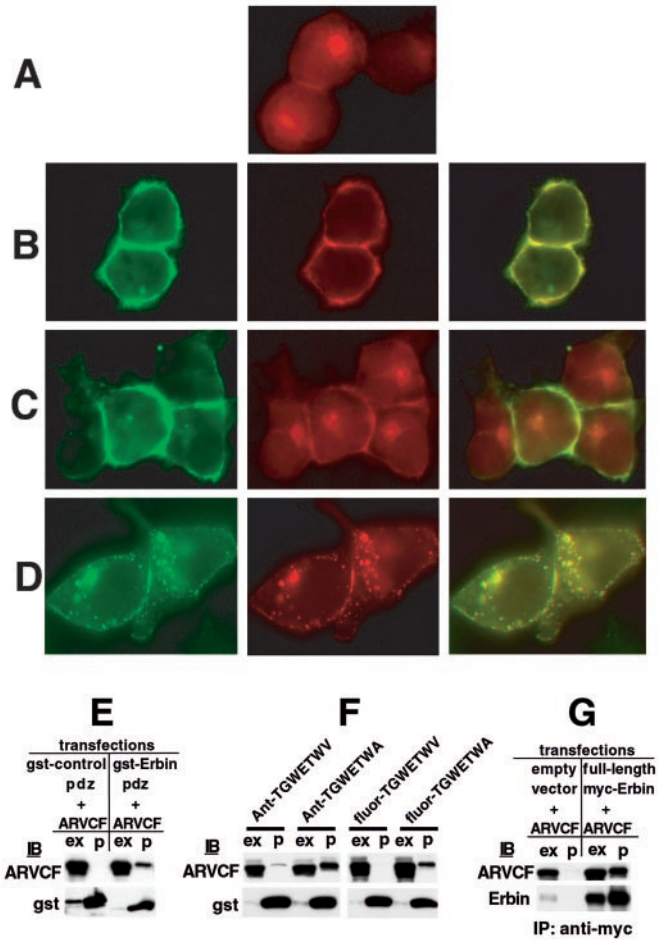

FIG. 2. **δ-Catenin and ARVCF form a stable complex with the Erbin PDZ domain when coexpressed in HEK 293 cells.** *A*, shown is the expression of the RFP-Erbin PDZ domain fusion protein in HEK 293 cells. The bright aggregates observed in the cytoplasm are a result of RFP, which forms identical structures when expressed alone. *B*, δ-catenin was coexpressed with the RFP-Erbin PDZ domain fusion protein. All RFP-Erbin PDZ domain fusion proteins, both cytoplasmic and that present in aggregates, were recruited to the cell junction by δ-catenin. *C*, δ-catenin Δc6 was coexpressed with the RFP-Erbin PDZ domain fusion protein. Although some co-localization was observed at the cell junction, most of the RFP-Erbin PDZ domain fusion protein was present in the cytoplasm or intracellular aggregates, similar to when the RFP-Erbin PDZ domain fusion protein was expressed alone. *D*, ARVCF was coexpressed with the RFP-Erbin PDZ domain fusion protein. *E*, ARVCF and the GST-Erbin PDZ or GST-MAGI-3 PDZ1 (control PDZ) domain fusion protein were cotransfected into HEK 293 cells. The ability of each PDZ domain to coprecipitate ARVCF from cell extracts was assessed using glutathione-Sepharose to precipitate the GST-PDZ domain fusions. Immunoblots were used to analyze 1% of the cell extracts (*ex*) or 30% of the precipitated protein (*p*). *F*, ARVCF and the GST-Erbin PDZ domain fusion protein were cotransfected into HEK 293 cells; and subsequently, the cells were treated with either a peptide phage-selected against the Erbin PDZ domain (TGWETWV-COOH) or an analogous peptide with a Val-to-Ala substitution at the C terminus (TGWETWA-COOH). The peptides were either attached to the Antennapedia peptide (*left panels*) or labeled with fluorescein (*right panels*). At 20 h post-transfection, the cells were incubated with peptides (35 μM) for an additional 6 h (Antennapedia conjugates) or 24 h (fluorescein conjugates). Cells were then washed; extracts were made; and the ability of the peptides to disrupt *in vivo* complex formation between the Erbin PDZ domain and ARVCF was assessed by coprecipitation and immunoblotting, as described for *E*. *G*, ARVCF was transfected alone or with full-length Myc-tagged Erbin into HEK 293 cells. Immunoprecipitations (*IP*) were carried out on transfected cell extracts with anti-Myc monoclonal antibodies. Immunoprecipitated proteins were detected by immunoblotting (*IB*) using monoclonal antibodies against ARVCF (*upper panel*) or rabbit anti-Erbin polyclonal antibody (*lower panel*).

domain to the cell junction. The co-localization was observed in all coexpressing cells in several transfection experiments. Importantly, although a C-terminal six-amino acid truncation of δ-catenin (Δc6) still associated normally with the cell junction, the deletion resulted in a large decrease in the amount of the Erbin PDZ domain recruited to the cell junction (Fig. 2*C*). In fact, no co-localization was observed in ~90% of cells coexpressing δ-catenin Δc6 and the Erbin PDZ domain. Partial co-localization (as in Fig. 2*C*) was observed only in cells expressing the highest levels of δ-catenin Δc6 and the Erbin PDZ domain. Nonetheless, the observed partial co-localization in some cells may suggest that there is an additional weak interaction between the Erbin PDZ domain and δ-catenin that is independent of the C-terminal six amino acids of δ-catenin. Whether or not this is true, an intact C terminus was absolutely required for

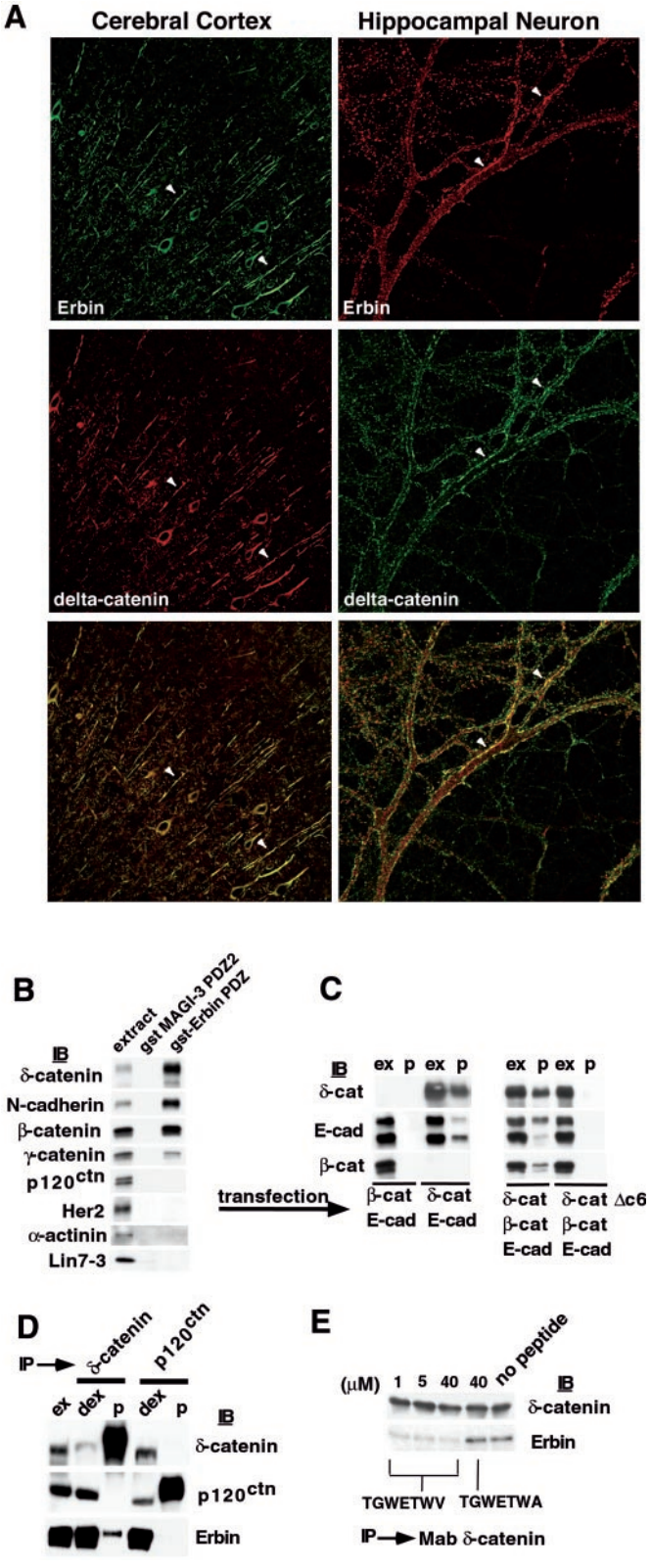

FIG. 3. **Endogenous interaction of Erbin and δ-catenin in brain.** *A*, cultured hippocampal neurons (*right panels*) or a section of rat cerebral cortex (*left panels*) was double-labeled with a monoclonal antibody directed against δ-catenin and polyclonal antibodies directed against Erbin to assess the level of co-localization for these proteins. *Arrowheads* point out dendritic shafts of pyramidal neurons in cortex (*left panels*) and dendritic shafts of cultured hippocampal neurons (*right panels*). *B*, *E. coli* cell-expressed GST-Erbin PDZ domain fusion protein (3 μg) and a control GST-PDZ domain fusion protein (3 μg) were assayed for the ability to precipitate, from mouse brain extracts, endogenous δ-catenin and proteins with which δ-catenin is know to associate. Precipitated proteins were analyzed by immunoblotting. *C*, HEK 293 cells were cotransfected or tri-transfected with the indicated constructs, and the ability of *E. coli* cell-expressed GST-Erbin PDZ domain fusion protein to precipitate δ-catenin-associated proteins was analyzed by immunoblotting (*IB*). The results show that the association of E-cadherin (*E-cad*) with δ-catenin was direct (*left panels*), whereas the association with β-catenin (*β-cat*) was indirect and required E-cadherin (*left and right panels*) and, finally, that precipitation of this complex by the Erbin PDZ domain required the C terminus of δ-catenin. *D*, δ-catenin and p120 catenin ($p120^{ctn}$) were immunoprecipitated with 5 μg of each antibody from 0.5 mg of mouse brain extract. An equal volume of the extract (*ex*) and the post-immunoprecipitation (*IP*) depleted extract (*dex*) and 50% of the precipitated protein (*p*) were analyzed by immunoblotting. Erbin co-immunoprecipitated only with δ-catenin, but not with the related family member p120 catenin. *E*, co-immunoprecipitation of Erbin with δ-catenin was disrupted by preincubating the brain extract with the Erbin PDZ domain-binding peptide TGWETWV-COOH, but not with the negative control peptide TGWETWA-COOH, at the indicated concentrations prior to immunoprecipitation. *Mab*, monoclonal antibody.

coprecipitation of δ-catenin with the Erbin PDZ domain in pull-down assays (Fig. 1*A*).

In contrast to the cell-surface localization of δ-catenin, ARVCF appears to be predominantly located in punctate cytoplasmic granules, with a lesser, discontinuous amount observed at the cell junction (Fig. 2*D*), consistent with the reported localization of the overexpressed protein in transfected fibroblasts (25). As with δ-catenin, all of the ARVCF completely co-localized with the Erbin PDZ domain. Thus, these data agree with the binding experiments described above, and they suggest that a potential function of δ-catenin and ARVCF *in vivo* is to recruit the Erbin protein to the cadherin-catenin complex via PDZ domain-mediated interactions.

We performed cotransfection and coprecipitation experiments to determine whether the interaction between ARVCF and the Erbin PDZ domain observed in Fig. 2*D* could be inhibited *in vivo* by a phage-derived peptide. Fig. 2*E* shows that ARVCF could be coprecipitated with the GST-Erbin PDZ domain fusion protein when both were coexpressed in HEK 293 cells. Fig. 2*F* illustrates that this interaction was inhibited when cotransfected cells were incubated for 6 h with a phage-derived peptide coupled to a fragment of the Antennapedia protein, but not when the cells were similarly incubated with a peptide in which the C-terminal Val residue was changed to Ala. Although the Antennapedia peptide is known to rapidly transport attached molecules across plasma membranes into the cell, we found that it was not necessary, as with a longer incubation, the same peptides coupled to fluorescein could also enter the cell and disrupt the Erbin PDZ domain/ARVCF interaction (Fig. 2*F*). These data suggest that binding between Erbin and δ-catenin or ARVCF *in vivo* requires an interaction between the PDZ domain and the C terminus of these proteins, respectively.

Finally, to determine whether binding of the Erbin PDZ domain to its C-terminal ligands is influenced by other domains within the full-length Erbin protein, we carried out a cotransfection and co-immunoprecipitation experiment with full-length Myc-tagged Erbin and ARVCF. Fig. 2*G* shows that ARVCF was precipitated by anti-Myc antibodies in the presence (but not absence) of full-length Myc-tagged Erbin. Coprecipitation of ARVCF with both the Erbin PDZ domain and full-length Erbin suggests that the binding characteristics of the Erbin PDZ domain are not directly regulated by other domains within the Erbin protein. The faint band visible in the *first lane* of the anti-Erbin immunoblot in Fig. 2*G* indicates that endogenous Erbin is present in HEK 293 cells. The presence of endogenous Erbin (and potentially ARVCF or p0071) did not affect the results of our experiments carried out in this

cell line. This is likely because of the relatively low expression levels of endogenous protein compared with the expression of proteins in transfected HEK 293 cells. This point is underscored by comparing the levels of endogenous Erbin with those of transfected Myc-tagged Erbin in HEK 293 cell extracts (Fig. 2*G*, *lower panel*).

Erbin Associates with the Cadherin-Catenin Complex in Brain—Both Erbin (26) and δ-catenin (30) are reportedly expressed in brain, so we chose this tissue to determine whether an Erbin-δ-catenin complex could be detected endogenously. Examination of adult rat cortex revealed that both proteins were present in the dendrites of the same population of cortical neurons (Fig. 3*A*, *left panels*). To examine whether these proteins co-localize at the subcellular level, hippocampal neurons were isolated, cultured for 21 days, and examined with antibodies directed against Erbin and δ-catenin. High magnification confocal microscopy analysis of these neurons revealed significant regions of co-localization (*white arrowheads*) on the plasma membrane of dendrites (Fig. 3*A*, *right panels*).

To examine whether the Erbin PDZ domain can associate with the endogenous cadherin-catenin complexes in brain, pull-down experiments were carried out with brain extracts, and proteins in the precipitate were analyzed by Western blotting with various antibodies. Fig. 3*B* illustrates that, as expected from previous experiments, the GST-Erbin PDZ domain fusion protein was able to efficiently interact with δ-catenin in brain extracts. δ-Catenin associated with the N-cadherin-β/γ-catenin complex in brain, and Fig. 3*B* also illustrates that these three proteins are found in the δ-catenin-Erbin PDZ domain complex. The specificity of this interaction is demonstrated by the observation that p120 catenin, which binds to cadherin in a manner mutually exclusive from δ-catenin, but which does not possess a PDZ domain-binding motif (20), was completely absent from the GST-Erbin PDZ domain precipitate (Fig. 3*B*). These data thus suggest that Erbin, δ-catenin, β-catenin, and N-cadherin can form an oligomeric complex. To discount the possibility that the Erbin PDZ domain also interacted directly with E/N-cadherin and/or β-catenin, we used cotransfection experiments to show that E/N-cadherin and/or β-catenin was precipitated only when δ-catenin was present and, even then, only when δ-catenin had an intact PDZ domain-binding motif (Fig. 3*C*). This is also consistent with ELISA experiments, in which the interaction of the PDZ domain-binding motif of β-catenin with the Erbin PDZ domain was barely detectable.

The GST-Erbin PDZ domain precipitate contained no detectable HER2, as one would expect based on the low affinity of the interaction between the HER2 C terminus and the Erbin PDZ domain. However, this result contrasted with the observation that detectable HER2 was precipitated from transfected HEK 293 cells. Upon further examination, we found that HER2 oligomerized into large complexes, as evidenced by the presence of disulfide-linked aggregates detected by immunoblot analysis of heterologously expressed HER2 under nonreducing conditions (data not shown). No such aggregates were detected with endogenously expressed HER2 in Caco-2 cells. The oligomerization of overexpressed HER2 resulted in the coupling of several low affinity C termini at close proximity, and it is likely that this served to artificially increase the apparent affinity of the HER2/Erbin PDZ domain interaction through avidity effects in the pull-down assay.

Finally, we showed that endogenous Erbin and δ-catenin could be co-immunoprecipitated from brain extracts (Fig. 3*D*). The specificity of this interaction is again underscored by the finding that immunoprecipitation of p120 catenin did not bring down Erbin. Importantly, the interaction of endogenous Erbin and δ-catenin could be disrupted by incubation of brain lysates with a phage-derived peptide, but not with a similar peptide containing a Val-to-Ala substitution at the C terminus (Fig. 3*E*). Together, these results suggest that Erbin is contained within a physiologically relevant cadherin-catenin complex in brain.

DISCUSSION

Our results show that δ-catenin and ARVCF are likely *in vivo* ligands of the Erbin PDZ domain. δ-Catenin, ARVCF, and p0071 are three p120-like catenins that share virtually identical PDZ domain-binding motifs and are the only proteins within the human genome that contain optimal Erbin PDZ domain-binding motifs predicted by C-terminal peptide phage display. *In vitro* and *in vivo* characterization of the interactions between the Erbin PDZ domain and ARVCF or δ-catenin showed that the interactions are specific and of sufficient affinity to be detected endogenously. Specifically, we have shown that this interaction occurs endogenously in neurons, but it is also likely to occur at the cell junctions of epithelia, where ARVCF and/or p0071 may be found (20). Our data also show that the previously reported interaction between the Erbin PDZ domain and HER2 (16, 31) is of low affinity compared with that of the Erbin PDZ domain and the p120-like catenins. However, we cannot rule out the possibility that this interaction is physiologically relevant based on the experiments presented here.

One possible function of the interaction between the Erbin PDZ domain and the C termini of the p120-like catenins is to recruit Erbin into a cadherin-catenin complex. Because there are several possible combinations that make up the cadherin-catenin complex in distinct cellular contexts, it is not surprising that three different catenins interact with Erbin. Although we have shown that Erbin can be integrated into this complex, the precise function of the different catenins and the functional consequences of these interactions remain to be elucidated. Possible functional consequences are further complicated by the existence of Densin-180, an isoform of Erbin (32). Erbin and Densin-180 are highly identical at the N terminus in the region of leucine-rich repeats, diverge significantly in the central region, and terminate with virtually identical PDZ domain sequences. While preparing this manuscript, we obtained results from a phage selection against the PDZ domain of Densin-180; the consensus sequence for the binding peptides was ETW(V/L)-COOH, which closely resembles the consensus-binding sequence for the Erbin PDZ domain. This suggests that Densin-180 likely associates with the cadherin-catenin complex as well. It is interesting to note that Erbin, ARVCF, and p0071 are widely expressed in many tissues, including brain (20), whereas δ-catenin and Densin-180 expression is restricted to brain (30, 32). Thus, even though all three catenins could likely bind both Erbin and Densin-180 PDZ domains *in vitro*, restricted expression patterns or subcellular localizations would dictate which interactions occur endogenously. Because our anti-Erbin antibodies were directed against an antigen largely consisting of the PDZ domain, it is possible that we actually detected Densin-180 rather than Erbin coprecipitating with δ-catenin from brain extracts. Even if this were the case, it would not alter the significance or interpretation of our findings that a LAP protein family member interacts with the cadherin-catenin complex in a physiologically relevant manner.

Two previously described genetic analyses highlight the possible functions for this class of protein/protein interactions. Analysis of mutations in *Drosophila melanogaster* that disrupt epithelial formation and growth control demonstrated that Scribble, a LAP protein containing four PDZ domains, appears to be a critical mediator of epithelial cell polarity (33–35). A

similar screen in *C. elegans* revealed that LET-413, a LAP protein that may be the nematode ortholog of mammalian Erbin, appears to also be involved in the assembly of the epithelial cell adherens junction (17, 18). Together, these data suggested that LAP proteins are involved in the appropriate assembly and polarization of epithelial cells. Mutations in either of these genes induce cytoskeletal changes in the affected epithelial cells, suggesting that one role for these proteins is to integrate cytoskeletal functions with epithelial cell morphology and polarization.

The importance of the link between the cadherin-catenin complex and the actin cytoskeleton is well established, and it is therefore significant that we have observed an interaction between the cadherin-catenin complex and a LAP protein that is related to both Scribble and LET-413. Interestingly, overexpression of δ-catenin in Madin-Darby canine kidney epithelial cells alters their morphology and promotes hepatocyte growth factor-mediated cell spreading, consistent with this protein and its interaction with Erbin having an effect on the cytoskeleton (29). Currently, we can only speculate on the mechanism by which this cytoskeletal regulation is accomplished. For example, the leucine-rich repeat regions in LET-413 and in Erbin are strongly homologous to a similar region in SUR-8/SOC-2, a protein that binds to and regulates the Ras GTPase (36, 37). Other Ras-related GTPases, particularly in the Rho/Rac class, are known to regulate the cytoskeleton (38). Thus, it is possible that LAP proteins help to unite the adherens junction-localized cadherin-catenin complex and the cytoskeleton by bringing cytoskeleton-regulating GTPases in close juxtaposition with this subcellular site. It is of particular interest that the gene *jac1* (GenBank™/EBI Data Bank accession number CAB60320), which encodes the most closely related *C. elegans* ortholog of δ-catenin and ARVCF, also terminates with the sequence DSWV-COOH. Furthermore, most residues within the Erbin PDZ domain predicted to interact with each of positions 0 through −3 by molecular modeling (9) are also conserved in the PDZ domain of LET-413. This suggests that mutation of *jac1* may yield a similar phenotype to that of LET-413 and provides insight into the mechanism of its role in cell polarization. In summary, the work reported here introduces a new paradigm for signaling to the cytoskeleton by the cadherin-catenin complex via an interaction with LAP family proteins.

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