The Hemidesmosomal Protein Bullous Pemphigoid Antigen 1 and the Integrin β4 Subunit Bind to ERBIN

MOLECULAR CLONING OF MULTIPLE ALTERNATIVE SPLICE VARIANTS OF ERBIN AND ANALYSIS OF THEIR TISSUE EXPRESSION*

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The bullous pemphigoid antigen 1 (eBPAG1) is a constituent of hemidesmosomes (HDs), cell-substrate adhesion complexes in stratified epithelia. Although its COOH terminus interacts with intermediate filaments, its NH2 terminus is important for its recruitment into HDs. To identify proteins that interact with the NH2 terminus of human eBPAG1, we performed a yeast two-hybrid screen, which uncovered a protein belonging to the LAP/LERP (for LRR and PDZ domain) protein family with 16 NH2-terminal leucine-rich repeats and a COOH-terminal PDZ domain. The gene for this LAP/LERP protein comprises at least 26 exons located on the long arm of chromosome 5. In most human tissues, several transcripts were detected differing in the coding region situated upstream of or within the PDZ domain. One of the encoded variants was found to correspond to the recently described protein ERBIN. In yeast and in vitro binding experiments, ERBIN was shown to interact not only with eBPAG1 but also with the COOH-terminal region of the cytoplasmic domain of the integrin β4 subunit, another component of HDs. Antibodies raised against the COOH terminus showed that ERBIN is expressed in keratinocytes. In transfected epithelial cells the protein, however, was not localized in HDs but was either diffusely distributed over the cytoplasm or concentrated at the basolateral plasma membrane. Because ERBIN had been shown previously to interact with the transmembrane tyrosine kinase receptor Erb-B2, which in turn associates with the integrin β4 subunit, we suggest that ERBIN provides a link between HD assembly and Erb-B2 receptor signaling.

Bullous pemphigoid antigen 1 (epithelial BPAG1 or eBPAG1) is a component of hemidesmosomes (HDs), multimeric adhesion complexes promoting cell-substrate adhesion in stratified and complex epithelia. Ultrastructurally, these complexes appear as electron-dense structures in close contact with the basal cell membrane associated with the cytoskeleton [1, 2]. eBPAG1 was identified originally as an autoantigen in the autoimmune subepidermal blistering disorder of the skin called bullous pemphigoid [3, 4]. This protein is a member of a protein family involved in cytoskeletal organization, the plakins that also comprise desmoplakin, plectin, envoplakin, and periplakin (reviewed in Ref. 5). The structural organization of the plakins is similar, with a central coiled-coil domain responsible for dimerization flanked by two large globular end domains. Although the NH2 termini of plakins seems to mediate their recruitment to distinct plasma membrane sites [6–9], their COOH termini are implicated in their attachment to the intermediate filament cytoskeleton [7, 10, 11]. Specifically, cell transfection and yeast two-hybrid analyses have demonstrated that the last 768 residues of human eBPAG1 contain sequences important for their interaction with intermediate filaments [7, 12]. Consistent with this result, eBPAG1 knockout mice show discrete signs of blistering, most likely as a result of an impaired attachment of keratin-intermediate filaments to HDs leading to the mechanical fragility of basal keratinocytes [13]. These mice also develop neurological degeneration with dystonia and ataxia, which results from the concomitant inactivation of the neuronal isoforms of eBPAG1, nPBAG1, or dystonin, which differ from eBPAG1 exclusively at their NH2 termini [13, 14]. These variants, which contain an actin or a microtubule-binding domain [7, 15], link the actin, the intermediate filament, and the microtubule networks to each other. They are essential for the maintenance of the cytoarchitecture of neurons [7, 15]. The NH2-terminal domain of eBPAG1 is implicated in its recruitment to HDs. This region associates with the cytoplasmic domain

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The abbreviations used are: eBPAG1 and -2, epithelial bullous pemphigoid antigen 1 and 2, respectively; HD, hemidesmosome; FN-III, fibronectin III repeat and PDZ domain; NHK, normal human keratinocytes; Ab, antibody; HA, hemagglutinin; GFP, green fluorescent protein; BD, DNA-binding domain; SC, synthetic complete; EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT, reverse transcription; ORF, open reading frame; EGFP, enhanced green fluorescent protein; GST, glutathione S-transferase; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; DIG, digoxigenin; anti-ERBIN Ab, affinity-purified anti-ERBIN-COOH-terminal peptide IgGs; nt, nucleotide(s); AD, transcription-activation domain.

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of two transmembrane components of HDs, the bullous pemphigoid antigen 2 (BPAG2, also termed BP180) and the integrin β4 subunit (9). BPAG2 is a type II transmembrane collagenous molecule (16). Transfection and yeast two-hybrid experiments have shown that its cytoplasmic domain associates with the distal COOH-terminal half of the cytoplasmic domain of the integrin β4 subunit (17, 18) and with eBPAG12 (9). The large collagenous extracellular domain serves as a cell surface receptor for extracellular matrix proteins. The α6β4 integrin plays a key role in HD assembly and in cell adhesion (reviewed in Ref. 2).

The integrin tyrosine kinase receptor Erb-B2, which in turn associates with was shown previously to interact with the transmembrane kinase transmembrane receptor Erb-B2 (25). Because ERBIN via its PDZ domain with the COOH terminus of the tyrosine kinase of COOH-terminal 129A3 reacting with the nonapeptide hemagglutinin (HA) epitope (YPYDVPDYA) (Roche), the anti-green fluorescent protein (GFP) polyclonal Ab (CLON-TECH), species-specific Texas red- or fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Nordic Immunological Laboratories), fluorescein isothiocyanate-conjugated goat anti-human IgG (Sigma), and Texas red- or fluorescein isothiocyanate-conjugated donkey anti-rabbit IgG (Amersham Pharmacia Biotech).

*Yeast Two-hybrid Experiments—* Yeast strain PJ69–4A (a gift of P. James, University of Wisconsin, Madison WI), the genotype of which is MATA, trpl–1, leu2–3,112, ura3–52, his3–200, gal4, gal80, GAL2–ADE2, LYS2–GAL1–HIS3, met2–GAL7–lacZ, was used as host for all yeast two-hybrid experiments. The yeast two-hybrid screening of a human keratinocyte cDNA library (CLONTECH) with the NH2 half of human eBPAG1, residues 1–1593 fused to the GAL4-DNA binding domain (BD) in pAS2–1 (CLONTECH) as a bait, was performed as described previously (32).

*Yeast-two-hybrid analysis with two defined constructs in pAS2–1 or pACT2 (CLONTECH) was performed in two steps. After yeast transformation, two samples were selected on agar plates containing complete (SC) medium lacking leucine and tryptophane. For each transformation eight colonies were arrayed in 96-well microtiter plates and then transferred onto agar SC medium lacking leucine and tryptophane (positive control), SC medium lacking leucine and tryptophane without adenosine, and SC medium lacking leucine and tryptophane without histidine and supplemented with 2 mM 3-amino 1,2,4-triazole. Growth was estimated after a 5-day incubation at 30°C. Transactivation controls were systematically performed for each construct with the opposite vector without insert. Selected positive clones were further confirmed by colony-lift filter assay for β-galactosidase activity in the yeast strain Y190 as recommended by the supplier (CLONTECH).

*Cloning of the Entire Coding Sequence of the Newly Identified Protein—* The insert of the prey plasmid 67, isolated in the yeast two-hybrid screen of a human keratinocyte cDNA library with GAL4-BD-eBPAG11–1593 as a bait, was completely sequenced and compared with those in the GenBank™ data base. The 3’ end of EST N40602 was identical to the 5’ end of the insert of the prey plasmid 67, and its sequence was used to design the following primers: 1) 5’-CCTAAGTTCAACAGAACAAC; 2) 5’-GTCATGGTGCATGCATTAGT; and 3) 5’- ACATGGTGCATGCATTAGTTCATG. For a 5’-RACE with the kit from Life Technologies, Inc. (2.0 version) according to manufacturer protocol. Total RNA was purified from NHK cells grown in low Ca2+ (0.07 mM) medium with the SV total RNA isolation system (Promega). First strand cDNA synthesis was performed with 3 μg of total RNA/reaction. The amplicons were cloned and sequenced. Then, the same strategy as described above was applied for a second round of 5’-RACE. The 3’ end of EST N11032 was completely identical to the 5’ end of the longest 5’-RACE amplicon. The following three primers were designed based on the EST sequence and used in a second 5’-RACE: 1) 5’-TTCAACGAAACATTAGTCTCA; 2) 5’-TAGAGGATCTGGAGATTGTTTCAGAGTTTCTGCA; and 3) 5’-TAAAGGTCTTGATGTTTCTGCA. Sequencing of the cloned amplicons of the second nested PCR and data base analysis indicated that the translation initiation codon of the protein partially encoded by the insert of the prey plasmid 67 had been reached (see Fig. 1, A and B).

To clone the entire cDNA encoding the novel protein, RT-PCR (Superscript one-step RT-PCR system, Life Technologies, Inc.) was performed with total RNA (0.2-μg/25-μl reaction mixture) isolated from NHK cells with three sets of forward and reverse primers, 5’-TATGCATCAAACGAAGATTGCTTGTG and 5’-GGTTGAGAAGATTGCTTGTG. The PCR products were cloned into the mammalian expression vector pCDNA3 (Invitrogen) digested with EcoRV and completely sequenced. The three cDNA pieces were sequentially fused in pCDNA3 to obtain a full-length ORF. The cDNA sequence has been given the GenBank™ accession number AF276423. The isolated cDNA was later found to be a variant of ERBIN.

To attach a HA tag either at the NH2 or COOH terminus of the identified protein, an adaptor coding for the HA tag and introducing an

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2 J. Koster and B. Favre, unpublished results.

3 The abbreviation LAP is also commonly used for leucine aminopeptidase. Thus, we suggest the unequivocal abbreviation LERP to refer to the family of proteins containing leucine-rich repeats and PDZ domain(s).
Expression of HA-tagged fusion proteins. The expression plasmid pMT2 (Sander, The Netherlands Cancer Institute, Amsterdam) allowing the expression plasmids described in detail elsewhere (19) and subcloned into some restriction enzyme sites on the schematic presentation of human ERBIN cDNA with the position of above) and the reverse primer 5'-H11032 with the forward primer of the third set of RT-PCR primers (see above) and cloned into pcDNA3-HA1 digested with BsaI and a BglII site as well as an initiation and stop codon was inserted by transferring the novel protein at its NH2 and COOH terminus, respectively, flank the internal portion of the protein, the most of which does not resemble any other protein.

The COOH terminus of the identified protein, residues 932–1412, of the novel protein were amplified by PCR with F1 polymerase from variant 1 cDNA and cloned in frame with the COOH terminus sequence of glutathione S-transferase (GST) into pGEX-2T (Amersham Pharmacia Biotech) with EcoRI and XhoI (Novagen) was transformed with these constructs or the vector pGEX-2T without insert. Expression of GST or GST-fusion proteins was induced by adding 0.5 mM isopropyl-1-thio-β-D-galactopyranoside in the broth medium for 3 h. Bacteria were collected by centrifugation, resuspended in phosphate-buffered saline (PBS) supplemented with 1% Triton X-100 and 5 mM EDTA, and lysed by sonication. Purification of GST-fusion proteins was performed as described previously (19). The protein concentration was determined with the protein assay reagent from Bio-Rad using bovine serum albumin as a standard. For pull-down assays, glutathione-Sepharose beads (Amersham Pharmacia Biotech) with bound GST or GST-fusion proteins were equilibrated in CHAPS buffer (25 mM Heps, 150 mM NaCl, 5 mM MgCl2, and 0.5% CHAPS (w/v), pH 7.5, and specific non-binding sites were blocked by incubation with 1% heat-inactivated bovine serum albumin diluted in CHAPS buffer.

GST Pull-down Assays—COS-7 cells transiently transfected with pcDNA3-HA2-β4, pcDNA3-HA2-β4 and or pMT2-HA-eBPAG1 were lysed and scraped in ice-cold CHAPS buffer supplemented with protease inhibitors (10 μg/ml leupeptin, pepstatin, and aprotinin and 1 mM di-isopropyl fluorophosphate). Lysates were precleared by two incubations of 1 h at 4°C with 10 μg of GST immobilized on glutathione-Sepharose beads. Precleared lysates were then incubated for 2–16 h at 4°C with 200 pmol of GST-fusion proteins immobilized on glutathione-Sepharose beads equilibrated in CHAPS buffer. After five washes with CHAPS buffer, bound proteins were eluted in SDS sample buffer and analyzed by SDS-PAGE and Western blotting.

Semiquantitative Analysis of ERBIN Transcript(s) in Human Tissues—The level of expression of ERBIN transcripts in 24 human tissues was determined by using a human Rapid-Scan panel (OriGene Technologies). In each well of the 96-well microtiter plate, 25 μl of the following PCR reaction mixture was added: 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1 μM each (NCL) of 67 primers, 0.5 μM of a forward (5'-GTAGTGTGTCCTCCACAGCC) and reverse (5'-CCCCGG-CTTTTTTGTCACAGGT) primers, and 2.5 units of Taq polymerase (Life Technologies, Inc.). The PCR conditions were: 1 × 94°C for 30 s, 55°C for 30 s, 72°C for 2 min, and 1 × 72°C for 10 min in a GeneAmp 9600 cycler (PerkinElmer Life Sciences). At the end of the PCR, two control reactions, without template and with 10 fg of pcDNA3 plasmid containing the 3′ end of ERBIN cDNA, were performed with the same PCR reaction mixture (kept on ice) and cycling conditions.

Southern and Northern Blotting—The probe was labeled with digoxigenin (DIG) antigen using the PCR DIG labeling mix (Roche) with the forward and reverse primers 5′-TCCCCATTGTGTTCTCTCAGCAA and 5′-GAGGAAACTCTTCTGTACATGA, respectively, from a plasmid derived from the yeast two-hybrid prey plasmid 67 and lacking both primer sequences used in the semiquantitative analysis of the transcripts from a human Rapid-Scan panel (see above). Labeling and Southern blotting were carried out exactly as described by the manufacturer. The Southern blot was developed with nitroblue tetrazolium salt and 5–5-bromo-4-chloro-3-indolyl phosphate as substrates for the alkaline phosphatase coupled to the anti-DIG Ab. For Northern blotting, a 3.8-kilobase pair EcoRI fragment from ERBIN cDNA was used as template to synthesize by PCR an antisense DIG-labeled DNA probe with the reverse primer 5′-CCCCGGTTTTTTTGTCACAGGT. Northern blotting was carried out exactly as described by the manufacturer (Roche). The DIG-labeled probe was detected on the Northern blot with CDP-Star screen (Novagen) and washed in 0.1× SSC (5× SSC is 0.15 M sodium chloride and 0.15 M sodium citrate) 3 × 30 min, 2 × 10 min, and 1 × 5 min in a GeneClean 9600 cycler (PerkinElmer Life Sciences).

In Vitro Transcription/Translation—The different constructs in pcDNA3 or pcDNA3-HA1 were transcribed and translated in vitro with the kit TNT T7 quick-coupled transcription/translation system (Promega).
megas) as indicated by the supplier.

Production and Purification of Polygonal Antibodies—A peptide corresponding to the 16 COOH-terminal residues of the novel protein (NH$_2$-TQFNTVELIIVREVVCOOH) was synthesized, isolated to 70% purity, and conjugated to Keyhole Limpet hemocyanin with glutaraldehyde by the company Eurogentech (Belgium). Two rabbits were immunized with the prepared antigen and bled 3 weeks after the third immunization at Eurogentech. The antisera from one rabbit was purified with ammonium sulfate to 50% saturation (33), and IgG immunization was isolated from this fraction after dialysis by chromatography on Protein A-Sepharose CL-4B according to manufacturer protocol (Amer
dramatic by the company Eurogentech). Two rabbits were immunized

Binding of ERBIN to eBPAG1 and the Integrin β4 Subunit

| Exon no. | cDNA borders | Size |
|---------|--------------|------|
| 1       | 2–248        | 247  |
| 2       | 249–296      | 48   |
| 3       | 297–494      | 198  |
| 4       | 495–612      | 118  |
| 5       | 613–691      | 78   |
| 6       | 692–781      | 90   |
| 7       | 782–838      | 57   |
| 8       | 839–902      | 64   |
| 9       | 902–977      | 75   |
| 10      | 978–1122     | 145  |
| 11      | 1123–1195    | 73   |
| 12      | 1196–1325    | 130  |
| 13      | 1326–1441    | 116  |
| 14      | 1442–1511    | 70   |
| 15      | 1512–1611    | 100  |
| 16      | 1612–1733    | 122  |
| 17      | 1734–1907    | 174  |
| 18      | 1908–2093    | 186  |
| 19      | 2094–2208    | 115  |
| 20      | 2209–2392    | 93   |
| 21      | 2393–3938    | 1546 |
| 22      | 3939–4061    | 123  |
| 23      | 4062–4268    | 207  |
| 24      | 4269–4361    | 92   |
| 25      | 4362–4436    | 75   |
| 26      | 4437–5962    | 1526 |

results

Yeast Two-hybrid Screen with the NH$_2$ Half of eBPAG1 Identifies the Hemidesmosomal Proteins BPAG2 and the Integrin β4 Subunit as Interactors—The NH$_2$ terminals of desmoplakin and plectin mediate their recruitment to plasma membrane sites by interacting with transmembrane glycoproteins (6, 8, 19). It is therefore probable that the same region is responsible for the subcellular distribution of eBPAG1, which belongs to the same protein family. To identify proteins that interact with the NH$_2$-terminal half of eBPAG1, and may thus mediate its localization into HDs, we used the yeast two-hybrid system. Residues 1–1593 of eBPAG1 were fused to GAL4-BD in pAS2–1 to screen a human keratinocyte cDNA library in the PJ69–4A yeast strain. Upon selection of 3.3 million independent yeast colonies, 59 colonies grew on selective media lacking adenine and histidine. In agreement with previous studies that provided evidence for an interaction of eBPAG1 with the integrin β4 subunit and BPAG2 (9, 28), we identified several cDNAs for the cytoplasmic domain of the integrin β4 subunit among the positives. All of them encoded the COOH terminus of β4, the shortest starting at residue 1315 (GenBank™ accession no. CAA37656), indicating that this region is critical for the interaction with eBPAG1. Furthermore, one cDNA encompassing the entire cytoplasmic domain of BPAG2 was isolated. These findings provide additional support for the assumption that eBPAG1, by means of interactions with the cytoplasmic domain of both BPAG2 and the β4 subunit of the α6β4 integrin, connects the intermediate filament network to the basal plasma membrane (2, 9).

Isolation of ERBIN as Interactor of eBPAG1—Among the other clones identified in the yeast two-hybrid screen, two of them, clones 67 and 68, cored for an ORF of 481 residues corresponding to the COOH terminus of a novel protein. Because the partial sequence lacked an ATG start codon, we performed two 5’-RACE experiments using EST sequences to obtain a full-length ORF sequence (Fig. 1B). To construct a full-length cDNA clone, three fragments spanning each about one third of the ORF and containing a unique restriction enzymes site in their overlapping sequences were amplified by RT-PCR from total RNA isolated from human keratinocytes. Comparison of the sequence of the RT-PCR and 2 fragments with the 5’-RACE and EST sequences indicated a perfect match except for a stretch of 15 nucleotides (nt) (from nt 1611 to 1625), which was missing in the first 5’-RACE fragment (indicated by [caret] in Fig. 1B). Surprisingly, the third RT-PCR produced three distinct bands (see Fig. 1C). Cloning and sequencing of these bands revealed that the complexity was even greater (see below). The longest ORF, variant 1, which contains the 3’-end sequence of the original yeast two-hybrid clone 67, codes for a protein of 1412 residues with a predicted molecular mass of 158,238 Da. An initial Blast search (34) with the complete sequence identified a single protein with significant homology, rat densin-180 (35). The size of the proteins and the organization of their domains were strikingly similar. Protein sequence analysis (36) revealed the presence of two motifs at both extremities of the protein: 16 LRRs, each 23 amino acids long, close to the NH$_2$ terminus (from residues 23 to 391) and a PDZ domain at the COOH terminus (from residues 1321 to 1409) (Fig. 1D). Nevertheless, two observations did not support the hypothesis that the identified protein was the human homologue of rat densin-180: 1) the lack of homology in the middle part of the proteins (24% identity) in comparison with the LRRs (71% identity) and PDZ domains (68% identity) and 2) their different tissue expression (see below).

Shortly after we submitted the cDNA sequence of the identified protein in the GenBank™ data base (accession no. AF276423), the same protein was identified in a yeast two-hybrid screen of a mouse kidney cDNA library with the tyrosine kinase transmembrane receptor Erb-B2/HER2 as a bait (25). Borg et al. (25) cloned a full-length human cDNA (Gen-
Bank™ accession no. AF263744) that corresponds to our variant 2 (see below) and termed the protein ERBIN. Therefore, we will also use this terminology in the rest of the article.

**Chromosomal Localization of ERBIN**

Based on the partial draft sequence of the human genome, the ERBIN gene is located on the long arm of chromosome 5, reference interval D5S427-D5S647 (69.6–74.7 cM), physical position 322.02 cR3000 (GB4 map). ERBIN mRNA comprises at least 26 exons, the borders of which have been deduced by comparison with the consensus intron/exon and acceptor/donor sequences (37) (Table I).

**Characterization of Several Splice Forms of ERBIN that Differ in their Coding Sequence upstream of and within the COOH-terminal PDZ Domain**—Seven ERBIN variants have been identified thus far, and these variations are probably caused by alternate splicing between exons 21 and 26 (Fig. 2). Variants 2, 3, 6, and 7 differ from the longest isoform, variant 1, in a region situated upstream of the PDZ domain. Interestingly, variants 4 and 5, which lack exons 24 or 25, respectively, have a truncated PDZ domain. Finally, we also found a polymorphic difference at position 3909 A/G, leading to the amino acid substitution K1207E.

**ERBIN Is Expressed in Keratinocytes as is eBPAG1**—To assess whether ERBIN was expressed in the same keratinocytes that also express eBPAG1, we first performed Northern blot analysis. The ERBIN transcript was expressed as a doublet of 6900 and 7400 nt in human keratinocytes and the HaCat cell line, respectively (Fig. 3). Next, we performed Western blot analysis by using affinity-purified Abs directed against the COOH terminus of ERBIN, which recognizes the GST-ERBIN1307–1412 fusion protein (Fig. 4 A). When tested on NHK cell extracts, the anti-ERBIN Ab identified one major protein with an Mr of 200,000 in differentiated cells (Fig. 4 B), but only small amounts could be visualized in undifferentiated keratinocytes after long film exposures. The Mr is higher than the theoretical molecular mass deduced from the cloned cDNAs, which range from 146,019 to 158,238 Da (Fig. 2). However, when the cDNAs for various variants were translated in vitro and their products were separated on SDS-PAGE, the isoforms migrated with an Mr 180,000–200,000 (Fig. 4 C), suggesting that the above lower electrophoretic motility was an intrinsic property of the protein(s).

**Tissue Expression of ERBIN Transcripts**—To extend the data of the expression of ERBIN in man, we performed a semiquantitative PCR analysis with cDNA synthesized from total RNA isolated from 24 different tissues that had been normalized to obtain the same level of amplification of the β-actin transcript. Primers annealing close to the variable region of the ERBIN transcripts were chosen for the PCR to get further insight into the expression of the different variants. Southern blotting using a probe encompassing nt 3809–4540 (Fig. 1) was carried out to confirm that the PCR-amplified fragment(s) did indeed correspond to the ERBIN sequence. Based on the results presented in Fig. 5, different ERBIN

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**FIG. 2. Variants of ERBIN.** The sequence varying in the variants 2–7 (of decreasing length) in comparison with that of the variant 1 (the longest) is presented. On the extreme right hand side the exon (Ex) number (see Table I) is indicated. In most of the variants complete exons are missing. However, in variants 3 and 5 the exon 23 is truncated: nt 4107–4139 and 4062–4139 of exon 23 are missing, respectively. The theoretical molecular masses in Da of the ERBIN variants 1–7 are 158,238, 153,926, 152,586, 151,191, 150,956, 150,540, and 146,019, respectively, or, amino acids.
ERBIN Interacts with the Integrin β4 Subunit—The cytoplasmic tail of the β4 subunit is crucial for its interaction with various components of HDs and their formation (2, 39). Therefore, we tested the ability of the COOH terminus of ERBIN (residues 932–1412) to interact with the integrin β4 subunit in the yeast two-hybrid system. Growth was supported selectively when yeast was co-transformed with GAL4-AD-ERBIN932–1412 and GAL4-BD-β4 constructs, leading to the expression of the COOH terminus of the β4 integrin subunit. Specifically, the COOH terminus of ERBIN, GAL4-AD-ERBIN932–1412, interacted with β4 fragments containing either the third and fourth FN-III repeats and the extreme COOH terminus (residues 1457–1752) (GenBank™ accession no. CA97656) or the fourth FN-III repeat and the extreme COOH terminus (residues 1570–1752) of β4. No interaction was observed with the first two FN-III repeats and the connecting segment (residues 1115–1457) or with the COOH extremity of β4 (residues 1667–1752). Furthermore, as in eBPAG1, residues 932–1159 located upstream of the PDZ were essential for interaction with β4 (Table II).

ERBIN Forms a Complex with Either eBPAG1 or the Integrin β4 Subunit in Vitro—The ability of ERBIN to associate with β4 and eBPAG1 was tested in in vitro binding assays. Two COOH-terminal regions of ERBIN encompassing or not the PDZ domain (from residues 805 to 1179 and from residues 805 to 1412, respectively) were expressed as fusion proteins with GST (GST-ERBIN805–1179 and GST-ERBIN805–1412, respectively) and immobilized on glutathione-Sepharose beads. HA-tagged recombinant proteins of eBPAG1 (from residues 1 to 2161, HA-eBPAG11–2161) and of the β4 cytoplasmic domain (from residues 1115 to 1752 and from residues 1115 to 1328, HA-β41115–1752 and HA-β41115–1328, respectively) were expressed by transfecting COS-7 cells and used as fluid phase ligands. As illustrated in Fig. 6, recombinant HA-eBPAG11–2161 encompassing the NH2 terminus and the coiled-coil region of eBPAG1 and recombinant HA-β41115–1752 representing a large portion of the cytoplasmic domain of β4 bound to both GST-ERBIN805–1179 and GST-ERBIN805–1412 but hardly to GST alone. Furthermore, recombinant HA-β41115–1328, which lacks the region containing sequences critical for interaction with ERBIN in yeast, did not associate with any of the GST-fusion proteins tested.

Together, consistent with the yeast two-hybrid results, the COOH-terminal region of ERBIN, containing or not the PDZ domain, forms a complex with eBPAG1 and the β4 cytoplasmic domain.

Expression of ERBIN in Epithelial Cell Lines—The ORF sequence of the ERBIN identified in the yeast two-hybrid screening encoded residues 932–1412 was fused to the COOH terminus of EGFP to study the localization of the fusion protein in various cell lines: COS-7, 804 G, PtK2, HaCat, and PA-JEB-β4 keratinocytes. In all transfected cells, the EGFP-ERBIN932–1412 fusion protein was found either diffusely distributed over the entire cytoplasm (Fig. 7A) or at the lateral cytoplasmic membrane at sites of cell-cell contact, especially in PtK2 cells (Fig. 7E) and PA-JEB-β4 keratinocytes (Fig. 7C). However, in both 804 G cells (data not shown) and PA-JEB-β4 keratinocytes the expressed protein was not obviously colocalized with either the α6β4 integrin (Fig. 7, A–D) or eBPAG1 (data not shown), the staining pattern of which was characteristic for hemidesmosomal-like structures or stable anchoring contacts (17, 18). Finally, a nuclear staining was also occasionally observed. Similar results were obtained with full-length ERBIN (variant 2) HA-tagged at its COOH terminus (data not shown).

**Fig. 3. Northern blot analysis of ERBIN transcripts in human keratinocytes.** Total RNA (10 μg) isolated from human keratinocytes grown in low calcium (0.07 mM) (lanes 1) and high calcium (1.87 mM) media for 4 days after low to high calcium switch (lanes 2) or from HaCat cells (lanes 3) was size-fractionated on 1% formaldehyde agarose gels and stained with ethidium bromide (left half). The size in kilobases of eBPAG1, eBPAG11–887, and ERBIN932–1412 indicated that the PDZ domain of ERBIN and the coiled-coil region of eBPAG1 were deleted (Table II). Next, because PDZ-containing proteins (38), we assessed whether the association between ERBIN and eBPAG1 involved the PDZ domain. Two ERBIN cDNA fragments, coding for residues 932–1159 and 1169–1412, respectively (see Fig. 1), were fused in frame to the GAL4-AD sequence in the vector pACT2. In independent yeast two-hybrid experiments we first confirmed that the region of ERBIN encompassing or not the COOH terminal regions of ERBIN encompassing or not the PDZ domain (from residues 805 to 1179 and from residues 805 to 1412, respectively) were expressed as fusion proteins with GST (GST-ERBIN805–1179 and GST-ERBIN805–1412, respectively) and immobilized on glutathione-Sepharose beads. HA-tagged recombinant proteins of eBPAG1 (from residues 1 to 2161, HA-eBPAG11–2161) and of the β4 cytoplasmic domain (from residues 1115 to 1752 and from residues 1115 to 1328, HA-β41115–1752 and HA-β41115–1328, respectively) were expressed by transfecting COS-7 cells and used as fluid phase ligands. As illustrated in Fig. 6, recombinant HA-eBPAG11–2161 encompassing the NH2 terminus and the coiled-coil region of eBPAG1 and recombinant HA-β41115–1752 representing a large portion of the cytoplasmic domain of β4 bound to both GST-ERBIN805–1179 and GST-ERBIN805–1412 but hardly to GST alone. Furthermore, recombinant HA-β41115–1328, which lacks the region containing sequences critical for interaction with ERBIN in yeast, did not associate with any of the GST-fusion proteins tested. Together, consistent with the yeast two-hybrid results, the COOH-terminal region of ERBIN, containing or not the PDZ domain, forms a complex with eBPAG1 and the β4 cytoplasmic domain.

Transcripts are expressed in most human tissues; they seem particularly abundant in brain, heart, kidney, muscle, and stomach (Fig. 5, lanes 4, 5, 6, 12, and 13, respectively).

The PDZ Domain of ERBIN Is Not Necessary for the Interaction with eBPAG1—In independent yeast two-hybrid transformation experiments we first confirmed that the region of ERBIN from residues 932 to 1412, GAL4-AD-ERBIN932–1412, that was identified in the yeast two-hybrid transformation screen was able to interact with residues 1–1593 of eBPAG1, eBPAG11–1593. Furthermore, binding also occurred with a construct containing residues 1–887 of eBPAG1, eBPAG11–887, from which the sequences encoding the coiled-coil domain of BPAG1 were deleted (Table II). Next, because PDZ-containing proteins are known to mediate the interaction between proteins (38), we assessed whether the association between ERBIN and eBPAG1 involved the PDZ domain. Two ERBIN cDNA fragments, coding for residues 932–1159 and 1169–1412, respectively (see Fig. 1), were fused in frame to the GAL4-AD sequence in the vector pACT2. In independent yeast two-hybrid experiments, only the region of ERBIN encompassing residues 932–1159, which is located upstream of the PDZ domain and does not exhibit significant homology (21% identity) to the sequence of densin-180, was found to interact with both eBPAG11–1593 and eBPAG11–887 (Table II). These findings indicate that the PDZ domain of ERBIN and the coiled-coil domain of eBPAG1 are dispensable for their interaction.
In a yeast two-hybrid screen of a human keratinocyte cDNA library with the NH₂-terminal half of eBPAG1, we have identified the COOH terminus of a protein resembling rat densin-180 (35). Cloning of the full-length ORF demonstrated that this protein of 1412 residues with a predicted molecular mass of 180 kDa represents a proteolytic fragment of ERBIN. A DIG-labeled ERBIN cDNA fragment consisting of nt 3809–4547 amplifies a 4547 bp fragment (arrow). The same immunoreactive protein could also be detected in extracts from undifferentiated keratocytes after a long film exposure. The Ab also specifically detected a smaller protein (Mr of 67,000, star), which might represent a proteolytic fragment of ERBIN.

A cDNA for four ERBIN variants cloned in pcDNA3 were translated on an in vitro translation system (Fig. 2). Lane 2, the ERBIN probe hybridized to most if not all bands that could be seen in lane 1. The same primers and Southern blot analysis were used to study the expression of ERBIN variants in human tissues from Rapid Trans human panel (lanes 2–7). This panel consists of cDNA synthesized from total RNA isolated from 24 human tissues: lung, liver, adrenal gland, thyroid gland, colon, pancreas, testis, stomach, salivary gland, muscle, bone marrow, fetal brain, fetal heart, kidney, spleen, liver, lung, colon, small intestine, muscle, stomach, intestine, placenta, adrenal gland, pancreas, ovary, uterus, prostate, skin, and fetal liver. The amount of first strand cDNA from each tissue has been normalized using the level of β-actin as internal standard (OriGene). The results obtained with 2.5 ng of cDNA/well are presented. Lane 3 (PCR products separated on agarose gel and stained with ethidium bromide) corresponds to lane 4, which exhibited the most complex pattern. Except for the low size bands, most if not all the amplified bands correspond to ERBIN sequences, indicating that several variants are expressed in most human tissues.

**DISCUSSION**

In a yeast two-hybrid screen of a human keratinocyte cDNA library with the NH₂-terminal half of eBPAG1, we have identified the COOH terminus of a protein resembling rat densin-180 (35). Cloning of the full-length ORF demonstrated that this protein of 1412 residues with a predicted molecular mass of 158,238 Da has a domain organization identical to that of densin-180, with 16 LRRs toward the NH₂ terminus and a PDZ domain at the COOH terminus. However, both the low homology of their internal sequences and the very different tissue expression of their mRNA (35) indicate that these proteins are not orthologues. During the preparation of the article, one of the isolated variants, variant 2, was found to be identical to a very recently described protein, termed ERBIN. ERBIN was identified in a yeast two-hybrid screen of a kidney cDNA library with the tyrosine kinase transmembrane receptor Erb-B2/HER2 fused to GAL4-DB as a bait (25). Thus, through a yeast two-hybrid screen we have independently isolated ERBIN as interactor of eBPAG1.

The ERBIN gene is located on chromosome 5 within the reference interval D5S427-D5S647, in a region that has not yet been linked to a genetic disease. Northern blot analysis of the total RNA isolated from human keratinocytes demonstrated the existence of two major ERBIN transcripts, whereas RT-PCR experiments show the presence of several variants in most human tissues. Sequence analysis of seven variants indicates...
that they most likely derive from alternative splicing. These variants differ in a region situated upstream of and within the PDZ domain. Interestingly, the densin-180 gene is subject to similar alternative splicing, and its expression is developmentally regulated (40). However, it remains to be established whether all variants of the ERBIN mRNA are translated, especially those with a truncated PDZ domain. Only one variant appears to be expressed in differentiated human keratinocytes, whereas a doublet around M₉ 180,000 was systematically detected in several human tissues analyzed by Western blotting with anti-ERBIN Ab (25).

ERBIN belongs to a family of proteins called LAP/LERP that comprises, in addition to ERBIN (25) and densin-180 (rat) (35), Scribble (human and Drosophila) (41) and Let-413 (Caenorhabditis elegans) (42). The domain organization of all of them is strikingly similar: 16 LRRs and one or four PDZ domains at the NH₂ and COOH terminus, respectively (reviewed in Ref. 24). In addition to these two structural features, they all contain a short conserved region of 39 residues next to LRRs, termed the NH₂ and COOH terminus, respectively (25). In extension to these findings, our yeast two-hybrid results indicate that ERBIN has the potential to interact with the NH₂-terminal region of eBPAG1 and the cytoplasmic domain of the integrin β4 subunit. Sequences important for these associations reside within the globular end domain of eBPAG1 and probably the fourth FN-III repeat close to the COOH terminus of the β4 subunit. A region of ERBIN encompassing residues 932–1159 mediates binding to both proteins. These residues are located upstream of the variable region subjected to alternative splicing and the PDZ domain.

The ability of ERBIN to form a complex with eBPAG1 or the β4 integrin subunit was confirmed in in vitro binding assays. Recombinant HA-β4₁₁₁₅–₁₁₇₂ representing a large portion of the β4 tail and recombinant HA-eBPAG1₁₁₅₂–₂₁₆₁ containing the NH₂ terminus and coiled-coil domain of eBPAG1 bound to both the GST-fusion proteins, GST-ERBIN¹⁰⁵–₁₁₇⁹ and GST-ERBIN⁹⁰⁵–₁₁₄₂, whereas an HA-β4₁₁₁₅–₁₁₇₂ recombinant lacking the third and fourth FN-III repeats of the β4 tail did not. Consistent with our yeast two-hybrid experiments indicate that 1) the PDZ domain of ERBIN is not needed for the association with either the β4 integrin subunit or eBPAG1, and 2) sequences contained within the COOH-terminal half of the β4 integrin subunit tail are crucial for its association with ERBIN.

To assess the potential of ERBIN to become localized in HDs, we performed transient transfection studies. In various epithelial cells transfected with constructs encoding either full-length ERBIN or its COOH-terminal half, the distribution of the expressed proteins in the cytoplasm was diffuse. However, there...
was also a clear staining of the plasma membrane that was reminiscent of the distribution of endogenous ERBIN in colon epithelial cells (25). These findings indicate that the localization of ERBIN at the plasma membrane does not reflect an intrinsic property of the protein but most likely depends on its association with other proteins. In an immortalized keratinocyte cell line and in 804G cells that form HDs in culture, expressed ERBIN was not colocalized in HD-like structures, perhaps, for mechanisms regulating cell growth, differentiation, and migration.

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The Hemidesmosomal Protein Bullous Pemphigoid Antigen 1 and the Integrin β4 Subunit Bind to ERBIN: MOLECULAR CLONING OF MULTIPLE ALTERNATIVE SPLICE VARIANTS OF ERBIN AND ANALYSIS OF THEIR TISSUE EXPRESSION

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