Isolation of a Novel β₄ Integrin-binding Protein (p27BBP) Highly Expressed in Epithelial Cells*

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The integrin β₄ has a long cytodomain necessary for hemidesmosome formation. A yeast two-hybrid screen using β₄ cytodomain uncovered a protein called p27BBP that represents a β₄ interactor. Both in yeast and in vitro, p27BBP binds the two NH₂-terminal fibronectin type III modules of β₄, a region required for signaling and hemidesmosome formation. Sequence analysis of p27BBP revealed that p27BBP was not previously known and has no homology with any isolated mammalian protein, but 85% identical to a yeast gene product of unknown function. Expression studies by Northern analysis and in situ hybridization showed that, in vivo, p27BBP mRNA is highly expressed in epithelia and proliferating embryonic epithelial cells. An antibody raised against p27BBP COOH-terminal domain showed that all β₄-containing epithelial cell lines expressed p27BBP. This p27BBP protein is insoluble and present in the intermediate filament pool. Furthermore, subcellular fractionation indicated the presence of p27BBP both in the cytoplasm and in the nucleus. Confocal analysis of cultured cells showed that part of p27BBP immunoreactivity was both nuclear and in the membrane closely apposed to β₄. These results suggest that the p27BBP is an in vivo interactor of β₄, possibly linking β₄ to the intermediate filament cytoskeleton.

Interactions between basal lamina and cells are important in several phenomena including differentiation (1) and tumor progression (2). Integrins belong to a family of adhesion receptors expressed in most tissues. They are glycoprotein heterodimers formed by the noncovalent association of two subunits named α and β (3). Interference with integrin signaling is associated with a variety of effects, including regulation of gene expression and mitotic progression (reviewed in Ref. 4).

The integrin subunit β₄ associates with α₆ to form a multivalent laminin receptor (5–7). High levels of β₄ are found in most epithelia, in Schwann cells, and in several tumors of epithelial origin (reviewed in Refs. 8 and 9). The amino acid sequence of β₄ cytoplasmic domain is 1045 amino acids long and not homologous to any other β subunits, thus suggesting that β₄ has different cytoskeletal links and/or is coupled to a specific transduction pathway (10–13). In agreement with this, in squamous and transitional epithelia, β₄ is highly enriched in hemidesmosomes, specialized structures providing firm mechanical links between basal lamina and the intermediate filament cytoskeleton (14–16). Loss of function of β₄ both in human and in β₄−/− mice results in hemidesmosome disruption, blistering, and is lethal perinatally (17–19). The adhesive function of β₄ in hemidesmosome-free epithelia or Schwann cells is still largely obscure, as is its function in epithelial neoplasms.

Several lines of evidence indicate that the β₄ cytodomain mediates its function through the association with unknown cytoplasmic ligands. In particular, several cytodomain deletions impair the ability of β₄ to translocate into hemidesmosomes, and this involves also the absence of other hemidesmosomal components (17, 20–22). In vitro mutagenesis has restricted to a 303-amino acid stretch the region of β₄ necessary for its translocation to hemidesmosomes (20), and, within this 303-amino acid region, at least one mutation that leads to loss of function, in vivo, has been mapped (17). In cells that do not form hemidesmosomes, the transfection of the wild type portion of the cytosolic region of β₄ results in partial arrest in G₁ and apoptosis, an effect that is not observed when a β₄ mutant deleted in its cytosolic domain is transfected (23). Finally, following antibody ligation β₄ may recruit the Shc and Grb2 interactor molecules (21).

Taken together, these results strongly suggest that this part of β₄ associates with unknown cytosolic ligands to signal and be targeted to hemidesmosomes. Despite this, their identification has been difficult due to the relative insolubility of β₄ and of hemidesmosomal associated proteins. To bypass this problem, we have decided to isolate interactors of the β₄ subunit by the yeast two-hybrid system; we have constructed a vector encompassing the critical region of β₄ and screened an epithelial cDNA library. We have found that β₄ interacts with a previously unknown peptide, named p27BBP, that is segregated in two distinct pools: cytoplasmic and nuclear. Interestingly, p27BBP is highly expressed, in vivo and in vitro, in epithelial cells containing β₄, and is associated with the intermediate filaments fraction. These data suggest a role for p27BBP in linking β₄ to the intermediate filament cytoskeleton.

Experimental Procedures

Two-hybrid Screen—All the procedures employed are described in detail in Golemis et al. (24). LexA-β₄ and LexA-krB fusion proteins

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were prepared by subcloning PCR2 fragments of \( \beta \) and of trkB in the plasmid pEG202 (25). Following subcloning, the inserts were sequenced. All the plasmids were transfected in the EGY48 yeast strain to check for their ability to self-activate the reporter genes LEU2 and lacZ, and to enter the nucleus. To perform the interaction hunt, a HeLa cell cDNA library was isolated from Roger Brent, Harvard Medical School, and DNA was then transfected with the bait construct encompassing the first two fibronectin domains (FNIII) and the connecting sequence. A total of 1,350,000 independent colonies were obtained from the primary transformation.

The selection for interacting clones was performed in media containing galactose and lacking leucine. Surviving yeast colonies were further selected by plating them on 5-bromo-4-chloro-3-indolyl-\( b \)-galactoside/galactose-containing medium. Criteria for the subsequent selection of the clones are described under "Results." Plasmid cDNAs were rescued in the trypanophen-deficient bacteria strain KC8 and sequenced by dye terminator technique on an automated Perkin-Elmer sequencer.

**GST Fusion Protein Preparation, in Vitro Translation and in Vitro Binding Assay—** The GST fusion proteins were prepared by excising from the pGEX2T vector the coding regions of \( \beta \) and of trkB with EcoRI and NotI and subcloning them in the pGEXT4T1 vector (Pharmacia Biotech Inc.). All fusion proteins were prepared in the protease minus strain BL21. GST fusion proteins were purified on glutathione-Sepharose 4B beads according to the manufacturer's protocol. Before the interaction experiments, the amount of the fusion protein was determined by the standard protein quantitation assay.

In *vitro* translated \( p27^{\text{BBP}} \) was prepared by transcribing the full-length \( p27^{\text{BBP}} \) mRNA subcloned in the Bluescript vector with the CapScript kit (Boehringer Mannheim). The translation was subsequently performed with the wheat germ agglutinin extract from Promega, in the presence of radioactive methionine (Amersham Corp.). Conditions were as suggested by the manufacturer except for the final concentration of potassium acetate (40 mM).

The *in vitro* binding assay was performed as described by Swafield and Johnston (26). In the assay the desired amount of GST fusion protein was mixed with *in vitro* translated \( p27^{\text{BBP}} \) and incubated at 4 °C for 2 h. The interaction buffer included an Escherichia coli protein extract (10 mg/ml) in 50 mM potassium phosphate buffer, pH 7.5, 150 mM NaCl, 10% glycerol, 10 mM Triton X-100, 1 mM PMSF, and 10 mM benzamidine inhibitors. GST fusion protein complexes were then pooled down with glutathione beads and washed five times in the same buffer at 4 °C. The reaction products were solubilized in Laemmli buffer and run in 12% denaturing SDS-acrylamide gel. The gels were dried and exposed for 5 days.

**Screening of a Lambda-Zap Library, Sequence Analysis, and Cloning of Mouse Probes—** To obtain full-length clones of \( p27^{\text{BBP}} \), a human placental cDNA library (Stratagene) was screened using a 700-nucleotide probe spanning its COOH-terminal sequence. The screening was performed according to the manufacturer's instructions. One million plaques were screened, and double-positive plaques were identified. Plasmid DNA was recovered by *in vivo* excision and analyzed by restriction analysis. The two largest clones were sequenced in both strands. Sequence analysis was performed through Wisconsin Package Version 9.0, Genetics Computer Group (GCC), Madison, WI and through the sources present in the Internet (the main servers were: "pedro," the Baylor College of Medicine, the Flybase database, the EBI, the NCBI).

To clone the mouse homolog, primers spanning the most conserved domain of human \( p27^{\text{BBP}} \) and the EST clone F15081 (from Sus scrofa) were used to amplify cDNA from mouse tissues. Primer sequences are: Scro, 5' TACGGATCCGCATTCAAACTCCGA and Rat, 3' TGG GAAATTCATTGCTTGAACACACTCCGA, and Rat, 3' TGG GAAATTCATTGCTTGAACACACTCCGA. Following reverse transcription-PCR, a 400-base pair fragment was obtained, sequenced, and subcloned into the TA vector (InviGen). The accession number of the mouse partial sequence is Y11460.

**Southern Blot Analysis—** High molecular weight DNA was extracted following standard procedures (27). Ten \( \mu \)g of human placenta DNA were digested with restriction enzymes (Promega), electrophoresed on 0.8% agarose gel, denatured in alkali, neutralized, and transferred onto nylon membrane (Hybond-N, Amersham). The membrane was hybridized with random primed \( p27^{\text{BBP}} \) cDNA at 42 °C for 16 h in 50% formamide, 5% formic acid, 1% SDS, 0.5 M sodium phosphate, pH 7.0, and 1 · SSC. After hybridization, the membrane was washed once at room temperature in 2 · SSC, 0.1% SDS for 20 min. For low stringency washes, the filter was treated twice at 42 °C in 2 · SSC, 0.1% SDS for 20 min each time. For high stringency washing the filter was treated twice at 42 °C in 2 · SSC, 0.1% SDS for 20 min each time and in 1 · SSC, 0.1% SDS at 42, 50, and 60 °C for 20 min each time (28). Autoradiography was performed using Hyperfilm (Amersham) films at −70 °C for 8–16 h with intensifying screens.

**Northern Blot Analysis—** Total RNA from cell lines was extracted with the RNAzolB kit (Cinna/Biotex Laboratories). Total RNA from tissues were extracted by the guanidine thiocyanate phenol-chloroform method (29). Total RNA was reconstituted in deionized pyrocatechol-water and quantitated by optical density measurement and ethidium bromide staining. Ten \( \mu \)g of total RNA were electrophoresed on denaturing formaldehyde-agarose gels (28), transferred to Hybond-N filters, and processed according to the manufacturer's instructions. Hybridization was carried with 1 · 106 cpm of homologous random primer-labeled probe in 50% formamide at 42 °C. Conditions for hybridization and washing were as described previously (30). Filters were exposed for 1 week at −70 °C with intensifying screens. The filter of murine tissues mRNAs was probed with a glucose-6-phosphate dehydrogenase cDNA probe, and densitometric analysis was performed to normalize the results.

**In Vitro Hybridization —** Tissues from adult mice were fixed by perfusion in 4% paraformaldehyde. Mice embryos were fixed by immersion. Tissues were dissected out, postfixed in 4% paraformaldehyde overnight, and cryoprotected in ascending sucrose solutions. Following freezing in OCT, 10-μm sections were cryostat-cut and mounted on polylysine-coated slides. Hybridization was performed with a 1 · 35S in *vitro* translated species-specific antisense riboprobes (400 nucleotides long) or with a control sense probe. High stringency hybridization and washes were carried as described previously (31). Sections were dipped to NTB-2 emulsion (Eastman Kodak Co.), exposed 2 weeks, developed with Kodak D19, and counterstained with hematoxylin.

**Antibodies and Immunofluorescence—** A rabbit polyclonal antiserum against the COOH-terminal peptide of \( p27^{\text{BBP}} \) (NH2-CTIATSMRD-RI-D-galactopyranoside/galactose-containing medium. Criteria for the subsequent selection of the clones are described under "Results." Plasmid cDNAs were rescued in the trypanophen-deficient bacteria strain KC8 and sequenced by dye terminator technique on an automated Perkin-Elmer sequencer.

**Characterization of the Integrin-binding Protein \( p27^{\text{BBP}} \)**

1. The abbreviations used are: PCR, polymerase chain reaction; FN, fibronectin; PBS, phosphate-buffered saline; BBP, \( \beta \)-binding protein; GST, glutathione S-transferase; CLSM, confocal laser scanning microscopy.

2. The selection for interacting clones was performed in media containing galactose and lacking leucine. Surviving yeast colonies were further selected by plating them on 5-bromo-4-chloro-3-indolyl-\( b \)-galactoside/galactose-containing medium. Criteria for the subsequent selection of the clones are described under "Results." Plasmid cDNAs were rescued in the trypanophen-deficient bacteria strain KC8 and sequenced by dye terminator technique on an automated Perkin-Elmer sequencer.
NaCl. At the end of this procedure, cells are composed from a cytoplasmic and nuclear intermediate filament network containing keratins, lamins, and other partially characterized intermediate filament associated proteins. The fraction was controlled by blotting with anti-keratin antibodies (mouse monoclonal from Becton Dickinson, number 7650) and electron microscopy (courtesy of A. Villa (DIBIT, Milano, Italy), not shown).

Subcellular fractionation for nuclei and cytoplasm was performed according to Robbins et al. (34) with slight modifications. Epithelial cells (FG2 human pancreatic or A431 carcinoma cells) cultured in standard medium were washed three times with PBS and lysed by incubation in RSB lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EGTA, 10 mM sodium phosphate + protease inhibitors) at 4 °C. Cells were homogenized and subjected to five cycles of centrifugation and washes with RSB (375 g). The pellet (nuclear fraction) was resuspended, sonicated in lysis buffer containing Triton X-100, and cleared by centrifugation. The technique used for fractionation, as detected with specific enzyme markers, yields pure nuclei and 95% pure cytosolic fractions.

RESULTS

Interactors of β₄ Integrin—Mutational analysis has shown that a 303-amino acid segment encompassing the first two NH₂-terminal FNIII domains and the connecting sequence is necessary for mediating (i) β₄ incorporation into hemidesmosomes and (ii) signaling events (20, 21). To identify polypeptides that interact with the β₄ cytodomain, we made use of the yeast two-hybrid system. A 331-amino acid construct encompassing the first two FNIII domains and the connecting sequence, was used for the interaction hunt. The arrow indicates points where mutations that cause β₄ loss of function occur. The construct B was used in the second phase of the screening. Right, table showing the constructs prepared and their features. Note that most of the β₄ constructs behaved as transcriptional activators. B, interaction of p27BBP with β₄ in the yeast survival assay. Equal amounts of growing yeast cells were spotted on leucine-deficient medium and observed 48 h later. p27BBP interacts only with the bait (A), but neither with other FNIII domains of β₄ (B) or with unrelated molecules (C). The bait is unable to interact with the library vector (D). C, in vitro translated p27BBP binds β₄ in vitro (lane 4). In vitro translated p27BBP was mixed with control GST-trkB protein (5 µg, lane 1), beads (lane 2), 4 °C stored GST-β4 fusion protein (lane 3, see text), GST-β4 fusion protein (1 µg, lane 4). After 2 h at 4 °C the samples were precipitated with glutathione beads, denatured, loaded on a gel, and autoradiographed. The expected size of p27BBP is 27 kDa.

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Characterization of the Integrin-binding Protein p27BBP

p27BBP Interacts Specifically with the First Two FNIII Domains and the Connecting Sequence of p27BBP. Both in the Yeast System and in Vitro—p27BBP was isolated from two independent interaction hunts with β4, at an average frequency of 41 clones per million screened. In four independent retransfection experiments on yeast, p27BBP was found to interact exclusively with the first two FNIII domains and the connecting sequence of β4, but not with other part of the β4 molecule, including the last two FNIII domains or with control molecules (trkB-LexA). Southern blot analysis indicated that these clones were duplicates of the same cDNAs. They were sequenced and found to encode for a novel protein (named p27BBP, BBP = β4-binding protein) that was further characterized.

p27BBP Interacts with the First Two FNIII Domains and the Connecting Sequence of β4. Both in the Yeast System and in Vitro—p27BBP was isolated from two independent interaction hunts with β4, at an average frequency of 41 clones per million screened. In four independent retransfection experiments on yeast, p27BBP was found to interact exclusively with the first two FNIII domains and the connecting sequence of β4, but not with other part of the β4 molecule, including the last two FNIII domains or with control molecules (trkB-LexA fusion protein Fig. 1B). Sequence analysis of the shortest translational coding for p27BBP indicated that the COOH-terminal 135-amino acid sequence was necessary and sufficient for the interaction with β4.

The biochemical interaction between p27BBP and β4 was then studied in an in vitro system. GST fusion proteins of β4 and control GST were prepared and mixed in a liquid phase assay with in vitro translated full-length p27BBP (see below). In vitro translated p27BBP was found to bind a β4 fusion protein containing the first two FNIII domains and the connecting sequence (i.e. the bait), but neither GST control proteins, glutathione-agarose beads (Fig. 1C), or other parts of the β4 molecule (not shown). Approximately 15% of radioactive input p27BBP protein bound β4. Binding between β4 and p27BBP occurred rapidly (within minutes) and was resistant to high salt washing. The relative low levels (15%) of binding between β4 and p27BBP in the in vitro assay were due to the strong instability of the β4 fusion protein, as short term storage at 4 °C of β4 fusion protein resulted in rapid loss of binding (compare lanes 3 and 4 of Fig. 1C). This phenomenon has been recently observed for other β4 fusion proteins both in vivo and in other bacterial strains, and it has been suggested to be linked to the ability of β4 to bind calpain-like proteases (35).

Taken together, these data indicate that p27BBP can directly bind the β4 cytodomain in vitro.

p27BBP Is a Novel Evolutionary Conserved Protein Encoded by a Single Gene—The partial sequence of p27BBP lacked an ATG start codon and was not homologous to any known nucleotide sequence. To isolate full-length clones of p27BBP, a human placenta λ-Zap library was screened. From one-million clones, 50 positive plaques were rescued by in vivo excision and ana-
Characterization of the Integrin-binding Protein p27BBP

Fig. 3. Expression of p27BBP mRNA in mouse tissues and cell lines. A, Northern blot of 10 μg of total RNA extracted from mouse tissues. pla = placenta; ova = ovaries; bra = brain; ton = tongue; ski = skin; mus = muscle; lun = lungs; hea = heart; kid = kidney; tes = testis; spl = spleen; col = colon; duo = duodenum. This experiment is representative of four independent analyses. p27BBP mRNA levels in the different tissues have been normalized reprobing the filter with a glucose-6-phosphate dehydrogenase cDNA probe (graphic). p27BBP mRNA has an apparent size of 1.1 kilobase pairs (arrow). B, Northern blot of 10 μg of total RNA extracted from nonconfluent epithelial cell lines A431, FG2, and HeLa and from the activated T lymphocytes. Northern blot of 10 μg of total RNA extracted from nonconfluent epithelial cell lines A431 (A431 conf) and confluent overgrown A431 cells (A431 subconf). The ethidium bromide staining of the nylon sheet is shown for comparison.

p27BBP Is a Protein Highly Expressed in Epithelial Cells That Contain β₄—Preliminary results showed that p27BBP mRNA is unstable, and expressed in several epithelial human tissues, with high levels in the colon (not shown). To extend the expression data to an experimental model, the mouse homolog of the human p27BBP was cloned by reverse transcription-PCR using DNA analysis programs (BLOCKS, MOTIFS, and PRODOME) without revealing any motif or homology to characterized proteins. Strikingly, FASTA and BLAST analyses identified homologies with several EST clones and with two genes encoding putative proteins in yeast and Drosophila (Fig. 2A). Alignment of these three proteins revealed more than 75% identity and up to 90% similarity.

The presence of a gene coding for p27BBP in yeast and Drosophila suggested the possibility that duplication events might have led to homologous genes in mammals. To test this possibility, Southern blot analysis of human DNA was performed at high (Fig. 2B) and low stringency (data not shown). Human genomic DNA digested with SacI, PstI, EcoRI, HindIII, and BamHI was subjected to Southern hybridization analysis using the p27BBP cDNA as a probe. Surprisingly, the results were consistent with a single-copy gene.

p27BBP mRNA is expressed in embryonic epithelial cells and in the proliferative compartment of the adult colon. In situ hybridization of p27BBP mRNA in the developing embryo (A, B) and in the adult mouse (C, D). In the embryonic mouse p27BBP mRNA is highly expressed in the skin (Fig. 5A, upper panel, dark field and 5B, bright field) and in the gut (Fig. 5A, lower panel, dark field). In the adult mouse p27BBP mRNA is observed at low levels in the crypts of the gut (Fig. 5C, bright field). No labeling is seen using a sense probe (Fig. 5D). The scale bar is 40 μm for B, C, and D and 110 μm for A.

p27BBP mRNA expression was also examined in human epithelial cell lines. Northern blot analysis of p27BBP mRNA in human cell lines revealed high levels of expression in the epithelial cell line A431 and lower levels in HeLa and FG2 cell lines (Fig. 3B). All of these three cell lines contain high levels of β₄ mRNA and protein (not shown and Fig. 5). Steady state p27BBP mRNA level in epithelial cells was higher in subconfluent growing cells than in confluent cells (Fig. 3B). High expression levels were also seen in activated T cells (Fig. 3B).
To detect the cellular localization of p27\textsuperscript{BBP} mRNA, in situ hybridization studies were carried out, with particular attention to embryonic epithelial tissues expressing β\textsubscript{4}. In the developing mouse, p27\textsuperscript{BBP} mRNA was readily detected in developing epithelia, notably in the skin (Fig. 4A, upper) and in the primitive gut (Fig. 4A, lower). No labeling was seen using a control sense probe (not shown and Fig. 4D). In the embryos, p27\textsuperscript{BBP} mRNA was never restricted to the basal layer. By in situ hybridization, most murine adult tissues showed undetectable levels of p27\textsuperscript{BBP} mRNA. In the adult, p27\textsuperscript{BBP} mRNA was detected in skin keratinocytes and in gut epithelium (Fig. 4, C and D, and not shown). In these adult tissues, p27\textsuperscript{BBP} mRNA was either mainly basal, such as in the epidermis, or concentrated in the cells lining intestinal crypts.

Taken together the expression data show that p27\textsuperscript{BBP} mRNA is always expressed in all β\textsubscript{4}-containing epithelial tissues and cell lines and seems enriched in proliferating epithelia, such as in the embryonic skin. In addition, p27\textsuperscript{BBP} mRNA is also present at lower, but detectable, levels in tissues that have not been reported to contain β\textsubscript{4}.

The p27\textsuperscript{BBP} Protein Is Highly Insoluble, Associated with the Intermediate Filament Cytoskeleton Both in the Cytoplasm and in the Nucleus—To analyze the biochemical properties of p27\textsuperscript{BBP}, several approaches were tried. First, we tried to express the recombinant protein in heterologous systems such as E. coli; so far we have been unable to produce soluble p27\textsuperscript{BBP}. Second, a rabbit polyclonal antiserum was raised against the trated in the cells lining intestinal crypts. p27\textsuperscript{BBP}, several approaches were tried. First, we tried to ex-

![Image](325x489 to 546x729)

**Fig. 5.** p27\textsuperscript{BBP} immunoreactivity is present both in the cytoplasm and in the nucleus of epithelial cells containing β\textsubscript{4} integrin. A, FG2 cell line extract immunoprecipitated with the p27\textsuperscript{BBP} poly-

clonal antiserum (lane 1) or with preimmune serum (lane 2). The blot was then decorated with p27\textsuperscript{BBP} antiserum. Total extracts (30 μg) from FG2 cells blotted with the anti-p27\textsuperscript{BBP} antiserum (lane 3). The arrow points to the specific 27-kDa band recognized by the p27\textsuperscript{BBP} antiserum. B and C, coexpression of integrin p27\textsuperscript{BBP} and of β\textsubscript{4} in the A431 and FG2 epithelial cell lines. In B, total extracts of epithelial cells were run on 12% acrylamide gels and blotted with the p27\textsuperscript{BBP} antiserum; in C, total extracts of epithelial cells were run on 6% acrylamide gels and blotted with the 450-11A mouse monoclonal antibody directed against β\textsubscript{4}. The molecular mass of β\textsubscript{4} is 190 kDa. D, p27\textsuperscript{BBP} is present in two pools, one soluble and one associated with the intermediate filament fraction. FG cells were sequentially extracted in 1% Triton X-100 (soluble proteins, lane 1), ammonium sulfate-sucrose (cytoskeleton, lane 2), DNase I (histones and DNA binding proteins, lane 3), 2 M NaCl (loosely associated nuclear matrix proteins, lane 4). The pellets (lane 5) contain only proteins of the cytoplasmic intermediate/nuclear core filaments. Above, the same blot was reacted with an anti-keratin antibody (see “Experimental Procedures”) recognizing cytokeratins (8, 18, and 19 from the Moll’s Catalog, molecular masses ranging from 39,000 to 50,000 dal-

tons) as a control for the extraction procedure (E). p27\textsuperscript{BBP} is present both in the cytoplasmic fraction (SDS-solubilized extracts, lane 1) and in the nuclear fraction (lane 2). Fractons were prepared and controlled for their purity as described under “Experimental Procedures.” The blot was decorated with p27\textsuperscript{BBP} antiserum.

immunoprecipitation studies, some of p27\textsuperscript{BBP} was present in the Triton X-100-soluble fraction (Fig. 5D, lane 1), thus indicating that this protein exist in an equilibrium between a soluble and a highly insoluble form.

Since the intermediate/nuclear core filament fractionation method leads to the enrichment of both cytoplasmic filaments, composed in epithelial cells mainly of keratinins, and of nuclear core filaments, whose biochemical composition is largely unknown, we then performed fractionation studies between the nucleus and cytoplasm. Strikingly, p27\textsuperscript{BBP} was found present both in the nuclear and in the cytosolic insoluble fractions (Fig. 5E). The double localization of p27\textsuperscript{BBP} in the nucleus and in the cytoplasm was not due to cross-contamination of the two fractions, as shown by analysis with specific markers (not shown) and immunofluorescence studies (Fig. 6 and not shown).

To see the fine localization of p27\textsuperscript{BBP}, we analyzed by immu-

nnofluorescence and CLSM its expression pattern in epithelial cell lines. p27\textsuperscript{BBP} immunoreactivity was found in the cytoplasm and in the nucleus and could be abolished completely by preadsorbing the antiserum with the peptide (Fig. 6B and not shown). Cytoplasmic associated p27\textsuperscript{BBP} immunoreactivity ap-
The isolation of $\beta_4$-binding proteins has been a difficult task, despite the overwhelming evidence that its long cytoplasmic tail is involved in signal transduction and in hemidesmosome formation by interacting with unknown cytosolic proteins (17, 20, 21, 23). This is due both to the difficulty to solubilize $\beta_4$ and cytoskeletal proteins and to the different biological models employed. A further step in complexity is that, although $\beta_4$ is necessary for hemidesmosome formation, it is mostly expressed by epithelial cells that do not form hemidesmosomes, such as in the gut. At the present time, it is unknown whether $\beta_4$ binds the same cytoskeletal linkers in hemidesmosome-forming epithelial cells and in other epithelial cells, although the fact that mutations in the cytoplasmic domain of $\beta_4$ result in epithelial detachment both in skin and in the pylorus suggests the existence of a common cytoplasmic linker (17). Due to the fact that p27BBP seems to be tightly associated with the intermediate filament cytoskeleton, its interaction with $\beta_4$ may provide the long sought link between this integrin and the intermediate filaments both in epithelial cells that form hemidesmosomes as well in epithelial cells that do not form hemidesmosomes.

Circumstantial evidence, based on the available literature, would predict two types of molecules binding $\beta_4$: a tyrosine kinase (21) and an hemidesmosome/intermediate filament-associated protein (13, 20). In neither case, identification of these partners and evidence of direct association was so far obtained. The evidence of a tyrosine kinase associated with $\beta_4$ derived from immunoprecipitation with mild detergents followed by kinase assay and led to the model that a tyrosine kinase, not yet identified is intimately associated with $\beta_4$ (21). In this context, it is of interest to note that although our bait contained the motif of $\beta_4$ phosphorylated by the kinase, no tyrosine kinase could be identified in our two-hybrid screening. The possible explanations are that either $\beta_4$ does not associate directly with a kinase, that other parts of $\beta_4$ molecule are involved in the binding of the kinase, or that a posttranslational modification of $\beta_4$ is required to observe kinase association. The association of $\beta_4$ with cytoskeletal proteins is suggested by the fact that a deletion in the cytoplasmic first two FNIII domains causes the lack of $\beta_4$ association with hemidesmosomes and perturbs intermediate filaments organization. (17, 20). Four other major proteins of the hemidesmosome have been described: BPAG1, BPAG2, HD-1, and $\alpha_c$. $\alpha_c$ directly associates with $\beta_4$ to form a laminin receptor through a juxtamembrane domain not overlapping with our bait and thus explaining why no $\alpha_c$ was isolated in our screening. Interestingly, using the cytoplasmic domain of $\beta_c$, containing the first two FNIII domains as a bait, we did not observe any known hemidesmosomal proteins interacting with $\beta_c$. This is not surprising, as no molecule binding $\beta_c$ directly has ever been identified, but it is of some interest, since it has been recently reported that recombinant HD-1 may precipitate $\beta_4$ integrin from cell lysates (35). In this work (35), no evidence of direct binding of $\beta_c$ with HD-1 was produced, thus leaving open the possibility that this association occurs through intermediate molecules. The failure to isolate HD-1 in our two-hybrid screening clearly supports this latter possibility. Finally, the possibility that p27BBP is a component of the hemidesmosome will be clarified only by the generation of antibodies suitable for immunoelectron microscopy analysis or by the discovery of p27BBP mutants that target hemidesmosome assembly.

p27BBP, a Novel Intermediate Filament-associated Protein with a Broad Function—The difficulty encountered to solubilize p27BBP could have reflected the possibility that this protein was associated with cytoskeletal elements. In agreement with this observation, most of the p27BBP molecule was found to be present in the intermediate filament fraction. This finding

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**Fig. 6.** p27BBP is located both in the nucleus and at the basal aspect of the membrane of FG2 cells. CLSM z axis digital reconstructions of two FG2 cells stained for $\beta_4$ (red) and F-actin (green, A) and for p27BBP (red) and F-actin (green, B) show that a fraction of p27BBP is colocalized with $\beta_4$ in coherence with the submembranous F-actin cytoskeleton (indicated by triangles) as well as with nuclei (large red dots).
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raises several interesting questions that will require extensive investigations. The most important of these relates to the general function of p27\textsuperscript{BBP} in the absence of \(\beta_5\). Two facts are particularly intriguing: p27\textsuperscript{BBP} is already present in yeast cells and is highly identical to human p27\textsuperscript{BBP}. Strikingly, the identity is not limited to a specific domain, as it is scattered throughout the molecule and includes conservation of its length (245 amino acids in all species). This suggests that a strong evolutionary pressure is exerted on maintaining a tertiary moiety that is not limited to a specific domain, as it is scattered throughout the molecule and includes conservation of its length.

A second fact is the presence of p27\textsuperscript{BBP} in the nucleus. Recent extensive analysis has shown that also in the nucleus p27\textsuperscript{BBP} is present in the highly insoluble core filament fraction and is characterized by a striking redistribution during mitosis. In the past, ultrastructural data based on nonconventional electron microscopy techniques have shown that the nucleus contains a system of intermediate filament cytoskeleton that is not based on classical intermediate filaments such as keratins (39). One may speculate that p27\textsuperscript{BBP} is part of this intermediate filament cytoskeleton, whose biochemical composition is completely unknown. Interestingly, this core filament cytoskeleton is conserved from yeast to humans (39–41). The generation and analysis of yeast mutants will possibly clarify the function of p27\textsuperscript{BBP}.

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