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Isolation of a Novel β_4 Integrin-binding Protein ($p27^{BBP}$) Highly Expressed in Epithelial Cells*

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The integrin β_4 has a long cytodomain necessary for hemidesmosome formation. A yeast two-hybrid screen using β_4 cytodomain uncovered a protein called $p27^{BBP}$ that represents a β_4 interactor. Both in yeast and *in vitro*, $p27^{BBP}$ binds the two NH_2-terminal fibronectin type III modules of β_4, a region required for signaling and hemidesmosome formation. Sequence analysis of $p27^{BBP}$ revealed that $p27^{BBP}$ 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*, $p27^{BBP}$ mRNA is highly expressed in epithelia and proliferating embryonic epithelial cells. An antibody raised against $p27^{BBP}$ COOH-terminal domain showed that all β_4-containing epithelial cell lines expressed $p27^{BBP}$. The $p27^{BBP}$ protein is insoluble and present in the intermediate filament pool. Furthermore, subcellular fractionation indicated the presence of $p27^{BBP}$ both in the cytoplasm and in the nucleus. Confocal analysis of cultured cells showed that part of $p27^{BBP}$ immunoreactivity was both nuclear and in the membrane closely apposed to β_4. These results suggest that the $p27^{BBP}$ is an *in vivo* interactor of β_4, possibly linking β_4 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 β_4 associates with α_6 to form a multivalent laminin receptor (5–7). High levels of β_4 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 β_4 cytoplasmic domain is 1045 amino acids long and not homologous to any other β subunits, thus suggesting that β_4 has different cytoskeletal links and/or is coupled to a specific transduction pathway (10–13). In agreement with this, in squamous and transitional epithelia, β_4 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 β_4 both in human and in $\beta_4^{-/-}$ mice results in hemidesmosome disruption, blistering, and is lethal perinatally (17–19). The adhesive function of β_4 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 β_4 cytodomain mediates its function through the association with unknown cytoplasmic ligands. In particular, several cytodomain deletions impair the ability of β_4 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 β_4 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 β_4 results in partial arrest in G_1 and apoptosis, an effect that is not observed when a β_4 mutant deleted in its cytosolic domain is transfected (23). Finally, following antibody ligation β_4 may recruit the Shc and Grb2 interactor molecules (21).

Taken together, these results strongly suggest that this part of β_4 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 β_4 and of hemidesmosomal associated proteins. To bypass this problem, we have decided to isolate interactors of the β_4 subunit by the yeast two-hybrid system; we have constructed a vector encompassing the critical region of β_4 and screened an epithelial cDNA library. We have found that β_4 interacts with a previously unknown peptide, named $p27^{BBP}$, that is segregated in two distinct pools: cytoplasmic and nuclear. Interestingly, $p27^{BBP}$ is highly expressed, *in vivo* and *in vitro*, in epithelial cells containing β_4, and is associated with the intermediate filaments fraction. These data suggest a role for $p27^{BBP}$ in linking β_4 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-trkB fusion proteins

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y11435 (human complete sequence of $p27^{BBP}$) and Y11460 (mouse partial sequence of $p27^{BBP}$).

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were prepared by subcloning PCR[1] fragments of β_4 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 cDNA library (obtained from Roger Brent, Harvard Medical School) was co-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 β-D-galactopyranoside/galactose-containing medium. Criteria for the subsequent selection of the clones are described under "Results." Plasmid cDNAs were rescued in the tryptophan-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 pEG202 vector the coding regions of β4 and of trkB with *Eco*RI and *Not*I and subcloning them in the pGex4T1 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 Bradford protein quantitation assay (Pierce).

In vitro translated p27^{BBP} was prepared by transcribing the full-length p27^{BBP} mRNA subcloned in the Bluescript vector with the Cap-Scribe 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 Swaffield and Johnston (26). In the assay the desired amount of GST fusion protein was mixed with *in vitro* translated p27^{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 KCl, 1 mM $MgCl_2$, 10% glycerol, 1% Triton X-100, plus protease inhibitors. GST fusion protein complexes were 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 λ-Zap Library, Sequence Analysis, and Cloning of Mouse Probes—To obtain full-length clones of p27^{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 (GCG), Madison, WI and through the sources present in the Internet (the main servers were: "pedro," the Baylor College of Medicine, the Flybase data base, the EBI, and the NCBI).

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

Southern Blot Analysis—High molecular weight DNA was extracted following standard procedures (27). Ten μg 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 prime-labeled p27^{BBP} cDNA at 42 °C for 16 h in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0,5% SDS, and 20 μg/ml denatured sonicated salmon sperm DNA. 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 two times at 42 °C in 2 × SSC, 0.1% SDS for 20 min each time. For high stringency washing the filter was treated two times at 42 °C in 2 × SSC, 0.1% SDS for 20 min each time and in 1 × SSC, 0.1% SDS at 42, 50, 55, 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/Biotecx Laboratories). Total RNA from tissues were extracted by the guanidium thiocyanate phenol-chloroform method (29). Total RNA was reconstituted in diethyl pyrocarbonate-treated water and quantified by optical density measurement and ethidium bromide staining. Ten μg 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 × 10^6 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 reprobed with a glucose-6-phosphate dehydrogenase cDNA probe, and densitometric analysis was performed to normalize the results.

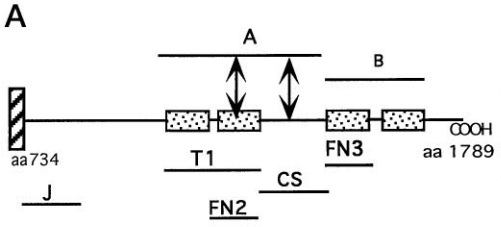
In Situ Hybridization—Tissues from adult mice were fixed by perfusion in 4% paraformaldehyde. Mice embryos were fixed by immersion. Tissues were dissected out, postfixed in 4% paraformaldehyde overnight, and cryoprotected in ascending sucrose solutions. Following freezing in OCT, 10-μm sections were cryostat-cut and mounted on L-polylysine-coated slides. Hybridization was performed with a ^{35}S *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^{BBP} (NH_2-CTIATSMRDSLIDSLT-COOH) was prepared by Eurogentec and tested for its specificity by Western blotting and immunoprecipitation both on recombinant *in vitro* translated p27^{BBP} and on cellular lysates. Integrin β_4 was detected with mouse monoclonal antibody 450-11A 10 μg/ml (a generous gift of Rita Falcioni, Istituto Regina Elena, Rome). Secondary antibodies were rhodamine-tagged swine anti-rabbit IgGs (1/50, Dako) and fluorescein-tagged goat anti-mouse IgGs (1/50, Antibodies, Inc.). In control experiments, primary antibodies were replaced by non-immune sera or irrelevant monoclonal antibodies. In addition, the p27^{BBP} antiserum was preadsorbed with the peptide used for its generation (1 μM, overnight, 4 °C). The immunofluorescence technique was as reported previously (16). Briefly, cell monolayers were fixed in 3% paraformaldeheyde, PBS, pH 7.6, 2% sucrose for 5 min at room temperature, permeabilized (3 min, 4 °C in 20 mM Hepes, 300 mM sucrose, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% Triton X-100, pH 7.4), and sequentially treated with blocking solution, primary and secondary antibodies (all diluted in PBS). Following washes, staining for F-actin was performed with fluorescein-labeled phalloidin (Sigma, 200 nM for 20 min at 37 °C in the dark). The coverslips were analyzed with a confocal laser scanning microscope (CLSM Bio-Rad 1024). Image files were recorded on different channels and digitally reconstructed to provide *z* axis views.

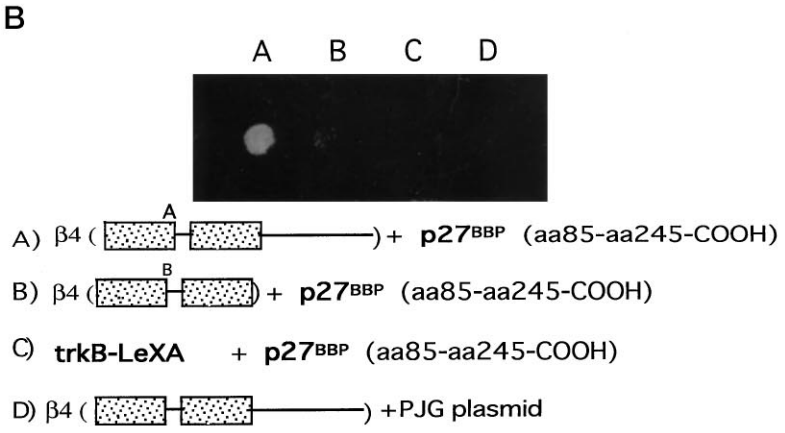
Fractionation, Immunoprecipitation, and Western Blot Analysis—Total extracts were prepared in boiling SDS (32). Immunoprecipitation from total extracts was performed diluting 1:10 the SDS extract in PBS followed by incubation with the p27^{BBP} antiserum, 1:1000, 2 h at 4 °C and subsequently with protein A-Sepharose, 30 min at 4 °C. Immunoprecipitates were washed five times in PBS, 1% Triton X-100 and loaded on denaturing 12% SDS-acrylamide gel. Cytosolic and nuclear extracts were run on denaturing 12% SDS-acrylamide gel (34) and transferred to Immobilon-P membranes (Millipore). The membranes were blocked with 5% bovine serum albumin in PBS and treated with p27^{BBP} antiserum used at 1:1000 dilution. Following washes and incubation with peroxidase-labeled anti-rabbit antibodies, the blots were developed by the chemiluminescence technique (Amersham).

Intermediate filaments/nuclear core filaments fractions were prepared exactly according to He (33). Briefly, all the soluble proteins, the non-intermediate filament cytoskeleton, DNA-associated proteins, and nuclear matrix proteins were removed by sequential washes in buffers containing Triton X-100, 250 mM ammonium sulfate, DNase I, and 2 M

[1] The abbreviations used are: PCR, polymerase chain reaction; FN, fibronectin; PBS, phosphate-buffered saline; BBP, β_4-binding protein; GST, glutathione *S*-transpherase; CLSM, confocal laser scanning microscopy.



Construct	Notes
A, 331 aa	Used as a bait
B, 206 aa	Used in selection tests
CS (connecting sequence), 143 aa	Strong transcriptional activator
T1 (first two FNIII), 189 aa	Moderate transcriptional activator
FN2 (second FNIII), 96 aa	Weak nuclear translocation
FN3 (third FNIII), 90 aa	Weak nuclear translocation
J (juxtamembrane sequence), 145 aa	No nuclear translocation



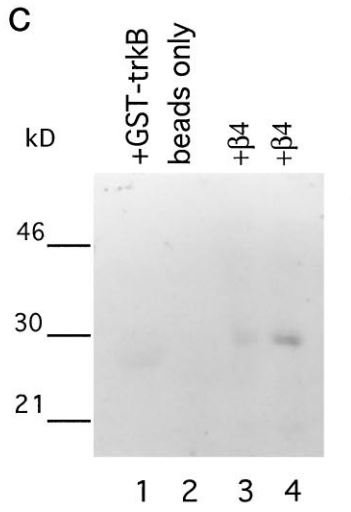


FIG. 1. *A*, organization of the cytosolic domain of the integrin subunit β_4 and constructs employed. *Left*, structure of the cytosolic domain of β_4 subunit. *Dotted rectangles* = fibronectin type III domains; *hatched rectangle* = transmembrane region. The *construct A* was used for the interaction hunt. The *arrow* indicates points where mutations that cause β_4 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 β_4 constructs behaved as transcriptional activators. *B*, interaction of p27^{BBP} with β_4 in the yeast survival assay. Equal amounts of growing yeast cells were spotted on leucine-deficient medium and observed 48 h later. p27^{BBP} 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 p27^{BBP} binds β_4 *in vitro* (*lane 4*). *In vitro* translated p27^{BBP} 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 p27^{BBP} is 27 kDa.

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 $MgCl_2$, 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 β_4 Integrin—Mutational analysis has shown that a 303-amino acid segment encompassing the first two NH_2-terminal FNIII domains and the connecting sequence is necessary for mediating (i) β_4 incorporation into hemidesmosomes and (ii) signaling events (20, 21). To identify polypeptides that interact with the β_4 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 joined to the DNA binding portion of the LexA repressor (β_4-LexA) and transfected into the EGY48 yeast strain (Fig. 1*A*). The construct-encoded protein was able to enter the nucleus and bind to LexA target sequences without showing transactivating activity. This construct containing the functional region of β_4 was used for the interaction hunt. Interestingly, most of the control constructs spanning the β_4 FNIII domain and the connecting sequence, when transported to the nucleus, were strong transcriptional activators *per se* and could not be used for any subsequent analysis in the yeast two-hybrid system.

To perform the interaction hunt, β_4-LexA was cotransfected with a translational fusion cDNA library from HeLa cells. HeLa cells express the integrin β_4 but do not normally assemble hemidesmosomes (not shown). Upon selection of 1-million independent yeast colonies in galactose-containing leucine-minus medium, approximately 300 colonies grew within 72 h after plating. Thus, the 331-amino acid domain of β_4, containing the FNIII domains, can interact with several proteins in the yeast two-hybrid system. Since published data indicated that a tyrosine kinase may be stably associated with β_4 (21), we first investigated whether a tyrosine kinase was present among our interactors. All the plasmid cDNAs were rescued from the hunt, and PCR with degenerate primers for tyrosine kinases

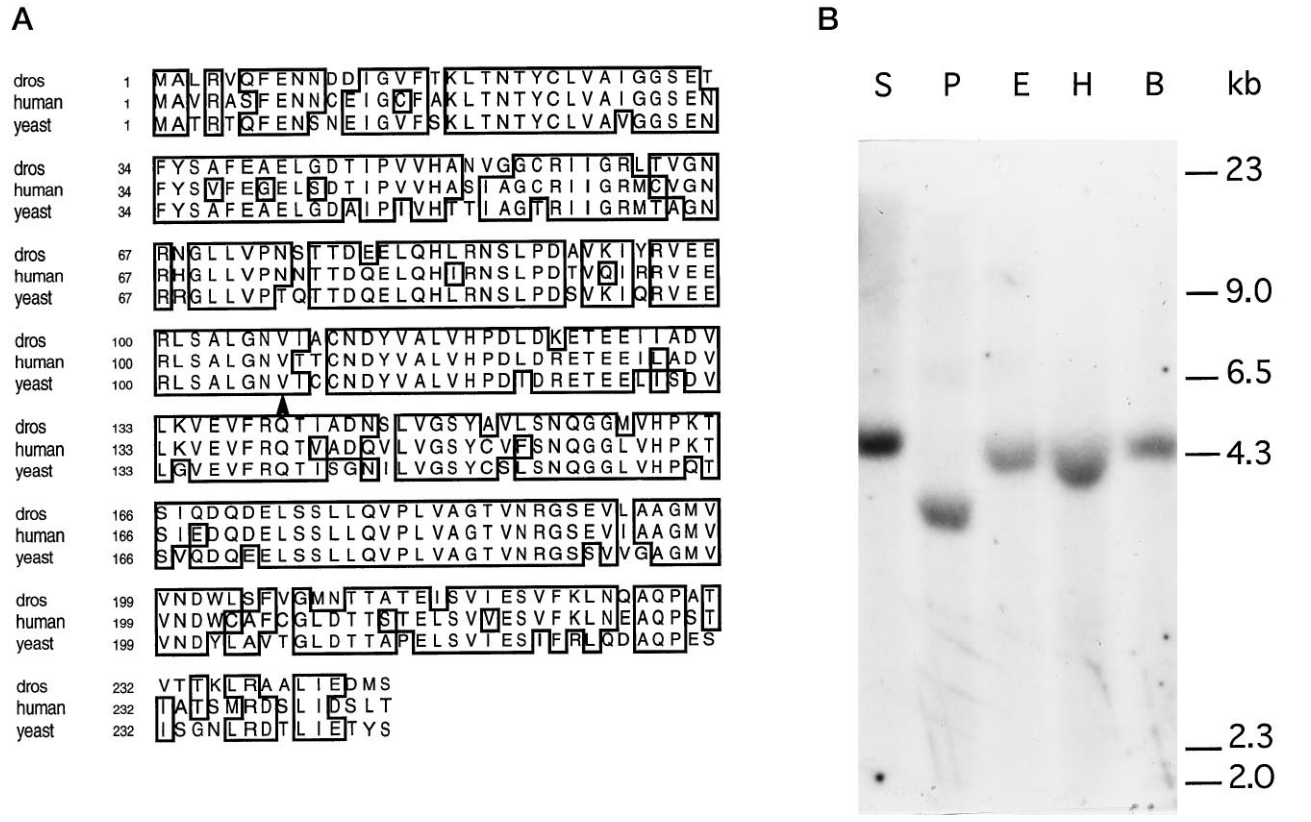


FIG. 2. *A*, sequence of p27^{BBP} protein and alignment with the *Drosophila* and yeast homologs. The predicted amino acid sequence of p27^{BBP} derived from the cDNA isolated from the placental library is shown. Alignment of human, yeast, and *Drosophila* sequences is shown. Accession numbers are Y11435 for human, X97641 for *Drosophila* (accession number X97641), and Z49919 for yeast. The *arrow* points to the beginning of the shortest clone interacting with β_4. The *boxed area* represents the regions of homology. *B*, genomic Southern analysis of the human p27^{BBP} gene. Placental genomic DNA was digested with *Sac*I (*S*), *Pst*I (*P*), *Eco*RI (*E*), *Hin*dIII (*H*), and *Bam*HI (*B*) and subjected to 0.8% agarose gel electrophoresis. The hybridization was carried out as described under "Experimental Procedures." The molecular weight markers were phage λ *Hin*dIII.

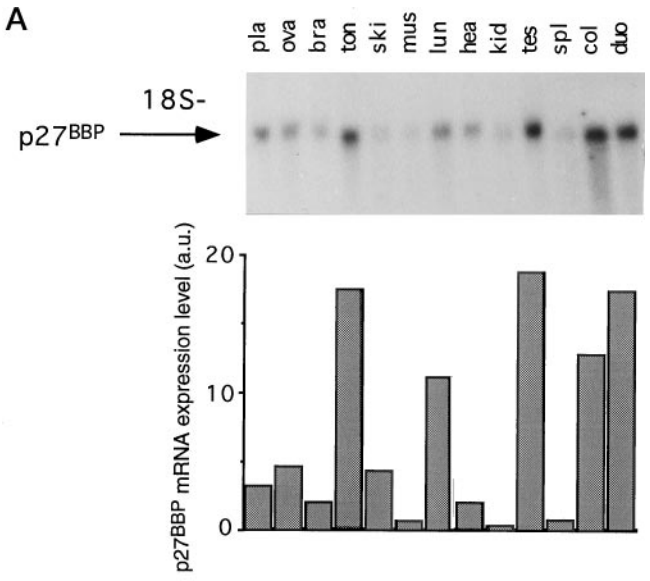
was performed: no evidence for tyrosine kinases associating with β_4 was found (not shown). To select for specific interactors of the functional domain of β_4, we then developed a test based on the screening of the ability of all the rescued plasmids to interact specifically with this region. All cDNA interactor plasmids were cotransfected either with the original bait or with a construct coding for the last two FNIII domains of β4 (that despite their high homology with the first two FNIII domains have been shown to be dispensable for β_4 signaling and its incorporation into hemidesmosomes; Refs. 17, 20, and 21) or with a control construct coding for trkB-LexA (trkB = cytodomain of truncated tyrosine kinase trkB). Most of the interactors bound to all FNIII domains of β_4, but not trkB-LexA. Around 50 clones interacted selectively with the first two FNIII domains and the connecting sequence of β_4 cytodomain, but neither with the last two FNIII domains nor with trkB. 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 p27^{BBP}, BBP = β_4-binding protein) that was further characterized.

p27^{BBP} Interacts Specifically with the First Two FNIII Domains and the Connecting Sequence of β_4 Both in the Yeast System and in Vitro—p27^{BBP} was isolated from two independent interaction hunts with β_4, at an average frequency of 41 clones per million screened. In four independent retransfection experiments on yeast, p27^{BBP} was found to interact exclusively with the first two FNIII domains and the connecting sequence of β_4, but not with other part of the β_4 molecule, including the last two FNIII domains or with control molecules (trkB-LexA fusion protein Fig. 1*B*). Sequence analysis of the shortest plasmid coding for p27^{BBP} indicated that the COOH-terminal 135-amino acid sequence was necessary and sufficient for the interaction with β_4.

The biochemical interaction between p27^{BBP} and β_4 was then studied in an *in vitro* system. GST fusion proteins of β_4 and control GST were prepared and mixed in a liquid phase assay with *in vitro* translated full-length p27^{BBP} (see below). *In vitro* translated p27^{BBP} was found to bind a β_4 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. 1*C*), or other parts of the β_4 molecule (not shown). Approximately 15% of radioactive input p27^{BBP} protein bound β_4. Binding between β_4 and p27^{BBP} occurred rapidly (within minutes) and was resistant to high salt washing. The relative low levels (15%) of binding between β_4 and p27^{BBP} in the *in vitro* assay were due to the strong instability of the β_4 fusion protein, as short term storage at 4 °C of β_4 fusion protein resulted in rapid loss of binding (compare *lanes 3* and *4* of Fig. 1*C*). This phenomenon has been recently observed for other β_4 fusion proteins both *in vivo* and in other bacterial strains, and it has been suggested to be linked to the ability of β_4 to bind calpain-like proteases (35).

Taken together, these data indicate that p27^{BBP} can directly bind the β_4 cytodomain *in vitro*.

p27^{BBP} Is a Novel Evolutionary Conserved Protein Encoded by a Single Gene—The partial sequence of p27^{BBP} lacked an ATG start codon and was not homologous to any known nucleotide sequence. To isolate full-length clones of p27^{BBP}, a human placenta λ-Zap library was screened. From one-million clones, 50 positive plaques were rescued by *in vivo* excision and ana-



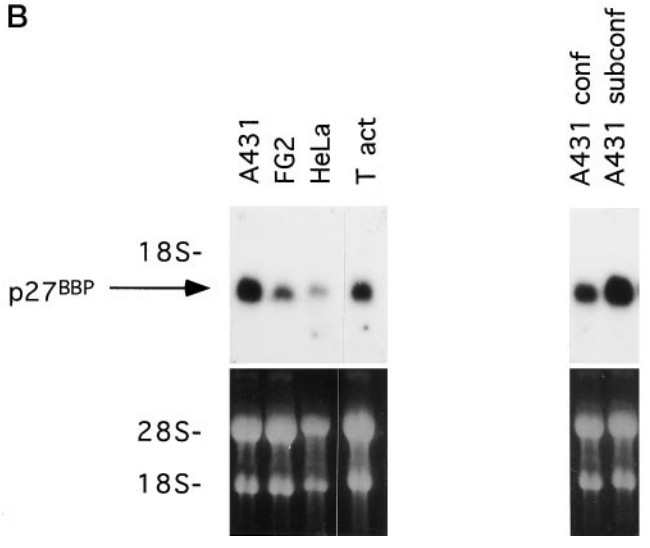


FIG. 3. **Expression of p27^{BBP} mRNA in mouse tissues and cell lines.** *A*, Northern blot of 10 μg of total RNA extracted from mouse tissues. *pla* = placenta; *ova* = ovaries; *bra* = brain; *ton* = tongue; *ski* = skin; *mus* = muscle; *lun* = lungs; *hea* = heart; *kid* = kidney; *tes* = testis; *spl* = spleen; *col* = colon; *duo* = duodenum. This experiment is representative of four independent analyses. p27^{BBP} mRNA levels in the different tissues have been normalized reprobing the filter with a glucose-6-phosphate dehydrogenase cDNA probe (*graphic*). p27^{BBP} 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 subconf*) and confluent overgrown A431 cells (*A431 conf*). The ethidium bromide staining of the nylon sheet is shown for comparison.

lyzed by restriction analysis. The size of the largest cDNA (1.1 kilobase pairs) was identical to the size of human p27^{BBP} mRNA (not shown and Fig. 3), contained a putative ATG initiation codon (36) and a 735-nucleotide open reading frame. The open reading frame predicts a 245-amino acid protein (expected molecular mass 27 kDa) with no signal peptides. The protein sequence was analyzed with 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. 2*A*). Alignment of these three proteins

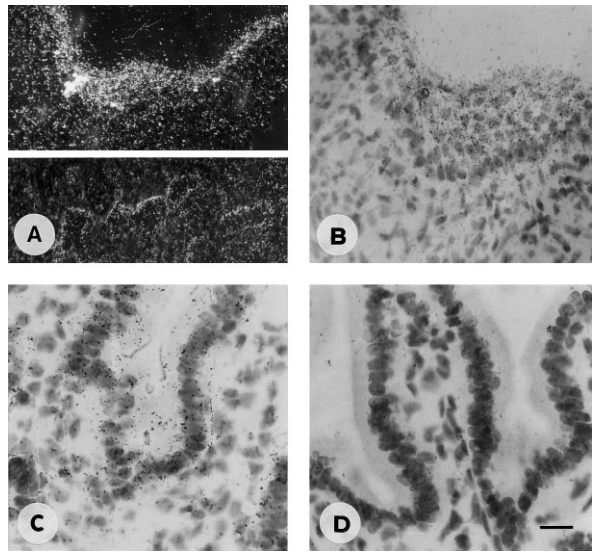


FIG. 4. **p27^{BBP} mRNA is expressed in embryonic epithelial cells and in the proliferative compartment of the adult colon.** *In situ* hybridization of p27^{BBP} mRNA in the developing embryo (*A, B*) and in the adult mouse (*C, D*). In the embryonic mouse p27^{BBP} mRNA is highly expressed in the skin (Fig. 5*A, upper panel*, dark field and 5*B*, bright field) and in the gut (Fig. 5*A, lower panel*, dark field). In the adult mouse p27^{BBP} mRNA is observed at low levels in the crypts of the gut (Fig. 5*C*, bright field). No labeling is seen using a sense probe (Fig. 5*D*). The *scale bar* is 40 μm for *B, C*, and *D* and 110 μm for *A*.

revealed more than 75% identity and up to 90% similarity.

The presence of a gene coding for p27^{BBP} 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. 2*B*) and low stringency (data not shown). Human genomic DNA digested with *Sac*I, *Pst*I, *Eco*RI, *Hin*dIII, and *Bam*HI was subjected to Southern hybridization analysis using the p27^{BBP} cDNA as a probe. Surprisingly, the results were consistent with a single-copy gene.

p27^{BBP} Is a Protein Highly Expressed in Epithelial Cells That Contain β_4—Preliminary results showed that p27^{BBP} 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 p27^{BBP} was cloned by reverse transcription-PCR using degenerate primers and used for analysis in adult mouse organs. Specifically, we concentrated on p27^{BBP} mRNA expression in epithelial tissues containing β_4 (all epithelial tissues, plus ovary and tongue) as compared with tissues that do not contain β_4 integrin (brain, skeletal muscle, hearth, kidney, testis, spleen). In adult mice, Northern blot analysis showed detectable levels of p27^{BBP} mRNA in all tissues analyzed. Among these tissues, some displayed remarkably high levels of p27^{BBP} mRNA. These included duodenum, colon, tongue, and lungs (Fig. 3*A*), that express high levels of β_4 integrin in their epithelial lining (Refs. 37 and 38 and not shown). Interestingly, high levels of p27^{BBP} mRNA were also detected in testis that has not been reported to contain β_4 integrin.

p27^{BBP} mRNA expression was also examined in human epithelial cell lines. Northern blot analysis of p27^{BBP} 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. 3*B*). All of these three cell lines contain high levels of β_4 mRNA and protein (not shown and Fig. 5). Steady state p27^{BBP} mRNA level in epithelial cells was higher in subconfluent growing cells than in confluent cells (Fig. 3*B*). High expression levels were also seen in activated T cells (Fig. 3*B*).

To detect the cellular localization of p27^{BBP} mRNA, *in situ* hybridization studies were carried out, with particular attention to embryonic epithelial tissues expressing β_4. In the developing mouse, p27^{BBP} mRNA was readily detected in developing epithelia, notably in the skin (Fig. 4*A, upper*) and in the primitive gut (Fig. 4*A, lower*). No labeling was seen using a control sense probe (not shown and Fig. 4*D*). In the embryos, p27^{BBP} mRNA was never restricted to the basal layer. By *in situ* hybridization, most murine adult tissues showed undetectable levels of p27^{BBP} mRNA. In the adult, p27^{BBP} mRNA was detected in skin keratinocytes and in gut epithelium (Fig. 4, *C* and *D*, and not shown). In these adult tissues, p27^{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^{BBP} mRNA is always expressed in all β_4-containing epithelial tissues and cell lines and seems enriched in proliferating epithelia, such as in the embryonic skin. In addition, p27^{BBP} mRNA is also present at lower, but detectable, levels in tissues that have not been reported to contain β_4.

The p27^{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^{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^{BBP}. Second, a rabbit polyclonal antiserum was raised against the COOH-terminal peptide of p27^{BBP} and used to study the distribution of endogenous p27^{BBP}. In agreement with the predicted size of the protein based on the cDNA sequence, by Western blot analysis this antiserum recognized a 27-kDa band in total lysates of wild type epithelial cells. Interestingly, in epithelial cells, a strong band was observed with as little as 10 μg of total extracts prepared in boiling SDS. This antiserum could also quantitatively immunoprecipitate p27^{BBP} from SDS-solubilized epithelial cells extracts (Fig. 5*A*). However, no immunoprecipitation of p27^{BBP} was seen using 1 milligram of protein extracted with mild detergents such as Brij 96, and only partial recovery was seen with 1 milligram of extracts prepared with 1% Triton X-100 (not shown). We then tried to establish whether p27^{BBP} could associate, *in vivo*, with β_4. Western blot analysis of total extracts of A431 or FG2 epithelial cell lines analyzed with either the anti-p27^{BBP} or an anti-β4 indicated that they expressed high levels of both p27^{BBP} and of β_4 (Fig. 5, *B* and *C*). Coimmunoprecipitation of β_4 and p27^{BBP} from FG2 and A431 epithelial cell lines was attempted by immunoprecipitating with a mouse monoclonal antibody directed against the extracellular domain of β_4, in detergent conditions that partially solubilize β_4 and/or p27^{BBP}, but may preserve protein-protein interaction (low SDS concentrations, Triton X-100, and Brij 96) followed by Western blot analysis with anti p27^{BBP}. Under these conditions no association could be detected.

The high insolubility profile of p27^{BBP} suggested that this protein could be part of the intermediate filament cytoskeleton, a relevant hypothesis, since β_4 is ultrastructurally linked to intermediate filaments (17, 18, 19, 35), but so far no molecule that directly binds β_4 and is associated with intermediate filaments has been found. We then performed fractionation studies on epithelial cells using a protocol that strips cells of all cellular components and leaves only an intermediate filament fraction that comprises both cytoplasmic intermediate filaments and the nuclear core filaments (33). Strikingly, p27^{BBP} was found to be highly enriched in the intermediate filament fraction (Fig. 5*D, lane 5*) and to be absent from all the other insoluble fractions (Fig. 5*D, lanes 2–4*). In agreement with

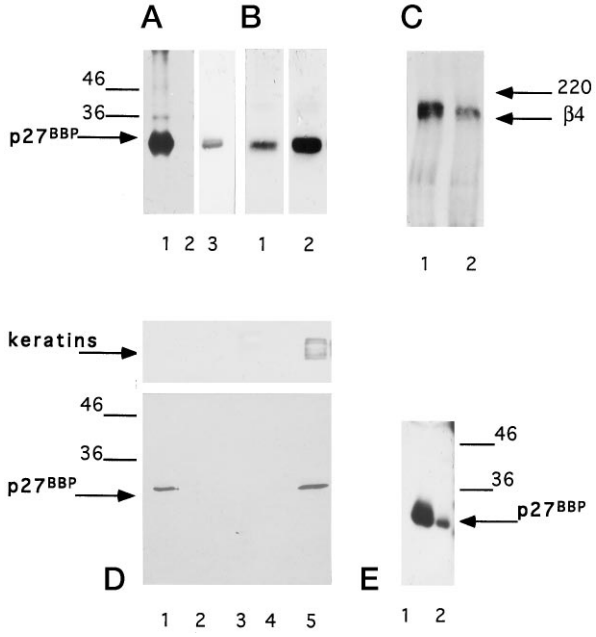


FIG. 5. **p27^{BBP} immunoreactivity is present both in the cytoplasm and in the nucleus of epithelial cells containing β_4 integrin.** *A*, FG2 cell line extract immunoprecipitated with the p27^{BBP} polyclonal antiserum (*lane 1*) or with preimmune serum (*lane 2*). The blot was then decorated with p27^{BBP} antiserum. Total extracts (30 μg) from FG2 cells blotted with the anti-p27^{BBP} antiserum (*lane 3*). The *arrow* points to the specific 27-kDa band recognized by the p27^{BBP} antiserum. *B* and *C*, coexpression of integrin p27^{BBP} and of β_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^{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_4. The molecular mass of β4 is 190 kDa. *D*, p27^{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 daltons) as a control for the extraction procedure (*E*), p27^{BBP} is present both in the cytoplasmic fraction (SDS-solubilized extracts, *lane 1*) and in the nuclear fraction (*lane 2*). Fractions were prepared and controlled for their purity as described under "Experimental Procedures." The blot was decorated with p27^{BBP} antiserum.

immunoprecipitation studies, some of p27^{BBP} was present in the Triton X-100-soluble fraction (Fig. 5*D, 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 keratins, and of nuclear core filaments, whose biochemical composition is largely unknown, we then performed fractionation studies between the nucleus and cytoplasm. Strikingly, p27^{BBP} was found present both in the nuclear and in the cytosolic insoluble fractions (Fig. 5*E*). The double localization of p27^{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^{BBP}, we analyzed by immunofluorescence and CLSM its expression pattern in epithelial cell lines. p27^{BBP} immunoreactivity was found in the cytoplasm and in the nucleus and could be abolished completely by preadsorbing the antiserum with the peptide (Fig. 6*B* and not shown). Cytoplasmic associated p27^{BBP} immunoreactivity ap-

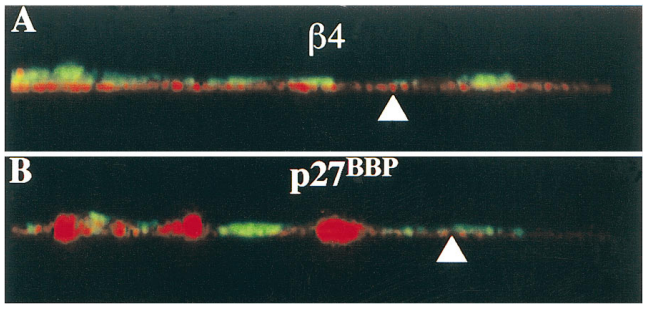


FIG. 6. **p27^{BBP} is located both in the nucleus and at the basal aspect of the membrane of FG2 cells.** CLSM *z* axis digital reconstructions of two FG2 cells stained for β_4 (*red*) and F-actin (*green, A*) and for p27^{BBP} (*red*) and F-actin (*green, B*) show that a fraction of p27^{BBP} is colocalized with β_4 in coherence with the submembranous F-actin cytoskeleton (indicated by *triangles*) as well as with nuclei (*large red dots*).

peared weaker in interphase cells than during mitosis (not shown). In addition, in human epithelial cells CLSM reconstruction of digitized images showed that p27^{BBP} was present at the immediate submembranous surface in a distribution consistent with β_4 (Fig. 6, *A* and *B*) and just adjacent to the subcortical cytoskeleton.

DISCUSSION

Through an extensive yeast two-hybrid screening, we have isolated p27^{BBP}, an interactor of the β_4 integrin. By means of two assays, *i.e.*the yeast two-hybrid system and *in vitro* binding, we have shown that p27^{BBP} binds to the region of the β_4 molecule necessary for signaling (17, 20, 21, 23). In addition, both *in vivo* and *in vitro*, p27^{BBP} has been found to be highly expressed in epithelial cells that contain β_4. In epithelial cells, p27^{BBP} is localized in the cytoplasm, mainly in the intermediate filament fraction, and at the submembrane level in close apposition with β_4. Taken together these data suggest that p27^{BBP} may be a novel cytoplasmic interactor of β_4 integrin, possibly linking it to the intermediate filament cytoskeleton.

In addition, various data indicate that p27^{BBP} has also a β_4 independent function: (i) part of p27^{BBP} is found in the nucleus, where to our knowledge β_4 has not been detected; (ii) the p27^{BBP} protein is highly conserved throughout evolution being expressed also in yeast, where no β_4 homolog has been yet described; (iii) expression data show that p27^{BBP} is also expressed in tissues where β_4 has not yet been detected.

Cytoplasmic Interactors of β_4 and the Possible Role of p27^{BBP}—Our paper provides the first evidence of a cytoplasmic molecule binding directly β_4 integrin. The possibility that p27^{BBP} and β_4 interact *in vivo* is supported by four lines of evidence: the specific association in the yeast two-hybrid assay, the direct association in the *in vitro* GST pulldown assay, the coexpression of p27^{BBP} and β_4 in epithelial cells *in vivo*, and the presence of p27^{BBP} immunoreactivity in epithelial cells at the submembrane level. It is important to stress that the binding of p27^{BBP} to β_4 occurs exclusively in the domain of β_4 that has been shown in several contexts, *in vitro* and *in vivo*, to be necessary for its function (7, 17, 20, 21, 23). However, so far, we were unable to coprecipitate β_4 and p27^{BBP}, and although preliminary experiments on cells transfected with p27^{BBP} seem to indicate a redistribution of β_4,[2] the relevance of this finding for the *in vivo* situation is still to be pondered. It is likely that the inability to coprecipitate β_4 and p27^{BBP} is due to the detergents needed to solubilize the cytoskeletal associated fraction of p27^{BBP} and that, under these conditions, the interaction between the two proteins is lost.

[2] S. Biffo, unpublished observations.

The isolation of β_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 β_4 and cytoskeletal proteins and to the different biological models employed. A further step in complexity is that, although β_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 β_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 β_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 p27^{BBP} seems to be tightly associated with the intermediate filament cytoskeleton, its interaction with β_4 may provide the long sougth 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 β_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 β_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 β_4 (21). In this context, it is of interest to note that although our bait contained the motif of β_4 phosphorylated by the kinase, no tyrosine kinase could be identified in our two-hybrid screening. The possible explanations are that either β_4 does not associate directly with a kinase, that other parts of β_4 molecule are involved in the binding of the kinase, or that a posttranslational modification of β_4 is required to observe kinase association. The association of β_4 with cytoskeletal proteins is suggested by the fact that a deletion in the cytoplasmic first two FNIII domains causes the lack of β_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 α_6. α_6 directly associates with β_4 to form a laminin receptor through a juxtamembrane domain not overlapping with our bait and thus explaining why no α_6 was isolated in our screening. Interestingly, using the cytoplasmic domain of β_4, containing the first two FNIII domains as a bait, we did not observe any known hemidesmosomal proteins interacting with β_4. This is not surprising, as no molecule binding β_4 directly has ever been identified, but it is of some interest, since it has been recently reported that recombinant HD-1 may precipitate β_4 integrin from cell lysates (35). In this work (35), no evidence of direct binding of β_4 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 p27^{BBP} 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 p27^{BBP} mutants that target hemidesmosome assembly.

p27^{BBP}, a Novel Intermediate Filament-associated Protein with a Broad Function—The difficulty encountered to solubilize p27^{BBP} could have reflected the possibility that this protein was associated with cytoskeletal elements. In agreement with this observation, most of the p27^{BBP} molecule was found to be present in the intermediate filament fraction. This finding

raises several interesting questions that will require extensive investigations. The most important of these relates to the general function of p27^{BBP} in the absence of β_4. Two facts are particularly intriguing: p27^{BBP} is already present in yeast cells and is highly identical to human p27^{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 molecular structure, possibly involved in multiple interactions with other proteins or in polymerization. At this stage sequence analysis does not allow any speculation on the mechanism of action of p27^{BBP}, since no motifs homologous to functional domains present in other proteins have been detected.

A second fact is the presence of p27^{BBP} in the nucleus. Recent extensive analysis has shown that also in the nucleus p27^{BBP} is present in the highly insoluble core filament fraction and is characterized by a striking redistribution during mitosis.[3] 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^{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^{BBP}.

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Isolation of a Novel β_4 Integrin-binding Protein ($p27^{BBP}$) Highly Expressed in Epithelial Cells

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