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# Epithelial Integrin $\alpha_6\beta_4$ : Complete Primary Structure of $\alpha_6$ and Variant Forms of $\beta_4$

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**Abstract.** The integrin  $\alpha_6\beta_4$  is a heterodimer predominantly expressed by epithelia. While no definite receptor function has yet been assigned to it, this integrin may mediate adhesive and/or migratory functions of epithelial cells. We have determined the complete primary structure of both the  $\alpha_6$  and  $\beta_4$  subunits from cDNA clones isolated from pancreatic carcinoma cell line libraries. The deduced amino acid sequence of  $\alpha_6$  is homologous to other integrin  $\alpha$  chains (18–26% identity). Antibodies to an  $\alpha_6$  carboxy terminus peptide immunoprecipitated  $\alpha_6\beta_4$  complexes from carcinoma cells and  $\alpha_6\beta_1$  complexes from platelets, providing further evidence for the association of  $\alpha_6$  with more than one  $\beta$  subunit. The deduced amino acid sequence of  $\beta_4$  predicts an extracellular portion homologous to other integrin  $\beta$  chains, and a unique cytoplasmic domain comprised of > 1,000 residues. This agrees with the structures of the  $\beta_4$  cDNAs from nor-

mal epithelial cells (Suzuki, S., and Y. Naitoh. 1990. *EMBO [Eur. Mol. Biol. Organ.] J.* 9:757-763; Hogervost, F., I. Kuikman, A. E. G. Kr. von dem Borne, and A. Sonnenberg. 1990. *EMBO [Eur. Mol. Biol. Organ.] J.* 9:765-770). Compared to these structures, however, the  $\beta_4$  cDNAs that we have cloned from carcinoma cells contain extra sequences. One of these is located in the 5'-untranslated region, and may encode regulatory sequences. Another specifies a segment of 70 amino acids in the cytoplasmic tail. Amplification by reverse transcription-polymerase chain reaction of mRNA indicated that multiple forms of  $\beta_4$  may exist, possibly due to cell-type specific alternative splicing. The unique structure of  $\beta_4$  suggests its involvement in novel cytoskeletal interactions. Consistent with this possibility,  $\alpha_6\beta_4$  is mostly concentrated on the basal surface of epithelial cells, but does not colocalize with components of adhesion plaques.

**T**HE interaction of cells with the extracellular matrix is important for the formation, maintenance, and repair of tissues as well as for other biological processes such as the metastasis of cancer cells. This interaction is mediated, in part, by a family of cell surface receptors called integrins (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Buck and Horwitz, 1987). These receptors form a link between the extracellular matrix and the cytoskeleton and may transmit signals from the extracellular to the intracellular environment that affect cell behavior.

Integrins are heterodimers comprised of  $\alpha$  and  $\beta$  subunits, that are noncovalently associated transmembrane glycoproteins. At least 11  $\alpha$  chains (Ruoslahti and Giancotti, 1989) and 6  $\beta$  chains (Sheppard et al., 1990) have been recognized in man. Each  $\alpha$  subunit tends to associate with only one type of  $\beta$  subunit, but there are several exceptions to this rule (Hemler et al., 1989; Cheresch et al., 1989; Holzmann et al., 1989; Freed et al., 1989).

We and others have previously reported the identification of an integrin expressed primarily on epithelial-type cells, termed  $\alpha_6\beta_4$  or  $\alpha_6\beta_4$  (Kajiji et al., 1987, 1989; Hemler et al., 1989). The alpha subunit,  $\alpha_6$ , also appears to associate with the  $\beta_1$  integrin subunit and, on platelets,  $\alpha_6\beta_1$  has been shown to function as a laminin receptor (Sonnenberg et al., 1988).

Here we report the complete cDNA sequence and the deduced amino acid sequence of both the  $\alpha_6$  and the  $\beta_4$  subunits. Both  $\alpha_6$  and  $\beta_4$  are homologous with the other integrin  $\alpha$  and  $\beta$  chains, respectively. However, they contain unique structural features which may suggest novel functional properties.

## Materials and Methods

### Cells

The human pancreatic carcinoma cell line FG was cultured as described previously (Kajiji et al., 1989) in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, and penicillin-streptomycin (50 IU/ml–50  $\mu$ g/ml). The human colon carcinoma cell line LoVo (Drewinko et al., 1976)

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was cultured in DME supplemented as above. Human platelets were the generous gift of Dr. Mark Ginsberg (Research Institute of Scripps Clinic, La Jolla, CA).

### Cell Labeling

FG cells ( $10^7$ ) were metabolically labeled with [ $^{35}$ S]methionine as described previously (Kajiji et al., 1989). Platelets were surface labeled with [ $^{125}$ I]sodium iodide and lactoperoxidase essentially as described by Roth (1975). Preparation of nonionic detergent cell extracts, immunoprecipitation, and analysis by SDS-PAGE have been described previously (Kajiji et al., 1989).

### Antibodies

The mouse monoclonal antibody S3-41 and the rabbit polyclonal antibody 5710 recognize  $\alpha_6\beta_4$  (Kajiji et al., 1987, 1989). The rat monoclonal antibody GoH3 (Sonnenberg et al., 1987), which is specific for  $\alpha_6$ , was the generous gift of Dr. Arnold Sonnenberg (University of Amsterdam, The Netherlands). The "anti- $\alpha_6$  cyto" rabbit antiserum was raised to a synthetic peptide (IHAQPSDKERLTSDA) corresponding to the carboxy terminus of  $\alpha_6$  based on the deduced amino acid sequence reported here. The monoclonal antibody, AA3, was produced using standard hybridoma procedures (Kohler and Milstein, 1975) from mice immunized with placental  $\alpha_6\beta_4$  purified on S3-41 affinity columns (Kajiji et al., 1989). AA3 has been shown to be specific for the  $\beta_4$  subunit by immunoprecipitation and Western blotting. Full characterization of this antibody will appear elsewhere (Cooper, H. M., and V. Quaranta, manuscript in preparation).

### cDNA Library Screening

Three different cDNA libraries were constructed (Invitrogen, San Diego, CA) from mRNA isolated from FG cells: an oligo-dT primed expression library in  $\lambda$ gt11 and two plasmid (pTZ18R Bst XI; Invitrogen) libraries, one oligo-dT primed, and the other random primed.

The following oligonucleotides were synthesized with a Gene Assembler (Pharmacia, Uppsala, Sweden) according to the amino-terminal sequence of the mature  $\alpha_6$  protein (Kajiji et al., 1989): (a) 40-mers with 64-fold redundancy covering the first 13 amino acids (FNLDTREDNVIRK)

5'-TTCAACTTAGACACGCGAGAGGACAACGTAATCCGAAAGT-3'  
C G T T G G

(b) 14-mers covering the complete redundancy of the first 5 amino acids

(1) 5'-TTTAATCTAGATAC-3'  
C C T C

(2) 5'-TTTAATCTGGATAC-3'  
C C C C

(3) 5'-TTTAATTTGGACAC-3'  
C C A T

Screening of the random-primed cDNA library was performed with the 40-mers, labeled with [ $\gamma$ - $^{32}$ P]ATP and T4 polynucleotide kinase (labeling kit from Pharmacia), using low stringency conditions (hybridization at 37°C overnight and washes with 2 $\times$  SSC at room temperature and at 46°C, 30-min each). 44 positives were then hybridized with the complete set of 14-mers followed by washes in 3.0 M tetramethylammonium chloride (TMAC) at progressively higher temperatures as described by Wood et al., (1985). Three clones,  $\alpha_6.1$ ,  $\alpha_6.31$ , and  $\alpha_6.44$ , remained positive up to the melting temperature of 14-mers (46°C).

The insert from the  $\alpha_6.1$  cDNA clone was isolated and used to screen the oligo-dT primed cDNA library. Preparation of probes, filters, hybridization, and washes were performed according to Maniatis et al. (1982).

The  $\lambda$ gt11 library ( $\sim 1 \times 10^6$  plaques) was screened (Young and Davis, 1983) with the rabbit antiserum 5710, which was raised against purified human  $\alpha_6\beta_4$  and previously shown by Western blots to react predominantly with  $\beta_4$  (Kajiji et al., 1989). 72 positive clones from the antibody screening were plaque purified and the cDNA inserts were amplified directly from bacteriophage plaques using the polymerase chain reaction (PCR).<sup>1</sup> Briefly, a plug of agar containing the plaque was transferred to 1 ml of SM (10 mM Tris-HCl pH 8.0, 50 mM NaCl, 5 mM MgCl<sub>2</sub>) and incubated for either 1 h at room temperature or overnight at 4°C. 10  $\mu$ l of supernatant

was used in a 50  $\mu$ l PCR containing 67 mM Tris-HCl pH 8.8, 1.5 mM MgCl<sub>2</sub>, 10 mM  $\beta$ -mercaptoethanol, 1.25 U of TAQ I polymerase, 0.25 mM each of dATP, dTTP, dCTP, and dGTP, and 0.1  $\mu$ g each of 24-mer oligonucleotides corresponding to sequences of  $\lambda$ gt11 flanking the Eco RI cloning site. The PCR program consisted of 3 steps: (a) one cycle at 94°C for 4 min; (b) 40 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C with a 5-per cycle extension on the 72°C segment; (c) 10 min at 72°C and a final shift to 4°C. These amplified fragments were isolated using Gene Clean (Bio 101, La Jolla, CA), digested with either Eco RI or Not I, repurified with Gene Clean, and subcloned into pKS+ (Stratagene, La Jolla, CA).

The 72 positive inserts were arranged into 11 groups based on cross-hybridization. Fusion proteins produced by clones representative of a group were used to select epitope specific antibodies (Weinberger et al., 1985) from the rabbit antiserum, 5710. These antibodies were tested for their ability to immunoprecipitate  $\beta_4$  from a SDS/heat-denatured FG lysate. The clone lam 18.2.1 (part of a group of 13 crosshybridizers) was identified as a  $\beta_4$  clone by this epitope selection method (Fig. 5 A). The insert from this clone was used to screen both of the plasmid cDNA libraries to isolate overlapping clones. Additional screenings were done using radiolabeled inserts from  $\beta_4$  positive cDNA clones until the complete  $\beta_4$  cDNA was isolated.

### DNA Sequencing

The  $\alpha_6$  cDNA clones were sequenced from restriction fragments (Pvu II or Sma I for  $\alpha_6.1$ ; Ava I + Bam HI or Eco RI + Hind III for  $\alpha_6.1-7$ ) subcloned into pKS+. The  $\beta_4$  cDNA clones were sequenced from nested deletion subclones created using a kit from Pharmacia. Both strands were sequenced by dideoxy chain termination (Sanger et al., 1977) either on an ABI 370A DNA Sequencer (Applied Biosystems, Inc. Foster City, CA) or manually (Sequenase kit; USB, Cleveland, Ohio) using either the T3 and T7 polymerase vector primer sequences or specific oligonucleotide primers synthesized to appropriate regions of the  $\alpha_6$  or  $\beta_4$  sequence. Sequences were analyzed on a VAX-VMS version 5.2 computer, with the programs of the University of Wisconsin Genetics Computer Group (Devereux et al., 1984).

### cDNA Synthesis and PCR

Poly-A+ RNA was isolated from human placenta and human carcinoma cells (Fast Track Kit; Invitrogen). 2–5  $\mu$ g was used to synthesize cDNAs with AMV reverse transcriptase (20 U; Molecular Genetics Resources, Tampa, FL) and 1  $\mu$ g of random hexamer primers (Pharmacia). The cDNAs were extracted with phenol/chloroform, precipitated with ethanol, and resuspended in 100  $\mu$ l of water. 1  $\mu$ l was amplified using the PCR conditions described above except for the primer concentrations (1  $\mu$ M each) and, for reactions using oligos 3 + 4 and oligos 5 + 6, the annealing temperature (52°C). The following oligonucleotides were used (see Fig. 6 for numbering; 5' nucleotide is in bold face type): (a) (82–98), (b) (310–329), (c) (4441–4482), (d) (4679–4697), (e) (4679–4697), and (f) (4805–4820). 10  $\mu$ l of the PCR mixture were separated on a 3.5% acrylamide gel along with 1 kb molecular size markers (Bethesda Research Laboratories, Gaithersburg, MD) and the DNA was visualized by staining with ethidium bromide.

To confirm the identity of PCR bands, these were subcloned and sequenced. Briefly one-half of PCR reaction was purified on glass beads (GeneClean, Bio 101) and ligated in the Sma I site of the vector pKS+ (Stratagene). Plasmids prepared from bacteria transformed with the ligation mixture were restriction digested and their inserts, if present, were sequenced in both directions as described above.

### Immunofluorescence

Cultured carcinoma cells were trypsinized, plated on coverslips coated with Matrigel (Collaborative Research, Lexington, MA) and cultured for 2 d. They were then fixed and stained with appropriate antibodies as described (Marchisio et al., 1984). Briefly, cells were fixed in 3% paraformaldehyde for 10 min at room temperature, permeabilized with the nonionic detergent Triton X100 (0.5%) for 5 min on ice, and then incubated with primary antibodies: mouse monoclonal AA3 specific for human  $\beta_4$ , control monoclonal Q5/13 specific for HLA-DR, a monoclonal antibody specific for vinculin (Sigma Chemical Co., St. Louis, MO), affinity-purified rabbit antibodies to talin (a gift from F. Giancotti, La Jolla Cancer Research Foundation, La Jolla, CA), protein A-purified anti- $\alpha_6$  cytoplasmic tail antibodies, control, irrelevant, rabbit immunoglobulin. Actin was stained with fluorescein isothiocyanate-conjugated phalloidin (Sigma Chemical Co.).

1. Abbreviations used in this paper: ORF, open reading frame; PCR, polymerase chain reaction.

Secondary antibodies were affinity purified goat anti-rabbit or anti-mouse immunoglobulins, conjugated to either fluorescein or rhodamine (Sigma Chemical Co.). Cells were viewed with a Zeiss Axiophot microscope equipped with an epifluorescence source, and photographed on Kodak T-max 3200.

## Results

### Isolation of cDNA Clones Encoding $\alpha_6$

We have previously isolated the integrin  $\alpha_6\beta_4$  from human carcinoma cells and human placenta and determined the amino-terminal sequence of both subunits (Kajiji et al., 1989). Based on the sequence of the amino terminus of  $\alpha_6$ , degenerate oligonucleotides were synthesized and used to screen  $3 \times 10^5$  colonies from a random primed human pancreatic carcinoma (FG) cDNA library as described in Materials and Methods. Three clones with inserts of  $\sim 1.2$  kb were isolated and sequenced. All three clones ( $\alpha 6.1$ ,  $\alpha 6.31$ , and  $\alpha 6.44$ ) overlapped and contained open reading frames (ORFs) whose deduced amino acid sequence matched exactly the protein sequence of the amino terminus of  $\alpha_6$  (Kajiji et al., 1989; Hemler et al., 1989).

To isolate the rest of the  $\alpha_6$  gene,  $\sim 7.2 \times 10^5$  colonies of the oligo-dT primed cDNA plasmid library was screened with the radiolabeled insert from the  $\alpha 6.1$  clone. Of 16 positive clones, one ( $\alpha 6.1-7$ ) had an insert of 4.8 kb. Overlapping restriction endonuclease fragments of the  $\alpha_6$  cDNA inserts were subcloned into pKS and sequenced in both orientations. Fig. 1 A shows the overall relationship of the  $\alpha_6$  cDNA clones to one another and the restriction sites relevant for subcloning and sequencing. The resulting sequence consists of 146 bp of the 5'-untranslated region, an ORF of 3,219

bp encoding 1,078 amino acids, and 2,264 bp of the 3'-untranslated region (Fig. 2).

### Analysis of the Primary Structure of $\alpha_6$

Preceding the amino terminus of the mature protein is a possible signal sequence of 23 amino acids. The mature protein is comprised of 1,055 amino acids with a  $M_r$   $\sim 117,000$ . The addition of carbohydrate with an average 2,500  $M_r$  to the protein core at the 10 potential N-glycosylation sites (N-X-S/T) would result in an estimated size of 142,000  $M_r$ . This relative molecular mass corresponds well with the 150,000  $M_r$  estimated from the migration of  $\alpha_6$  on SDS-polyacrylamide gels under nonreducing conditions.

A hydropathy profile (Kyte and Doolittle, 1982) of the deduced protein sequence identified a putative transmembrane region from amino acid residues 1,012-1,037. After this transmembrane region are 36 amino acids (residues 1,038-1,073) which comprise the presumed cytoplasmic domain. Analysis of the amino-terminal portion of the molecule revealed seven homologous repeat (domain I, residues 42-79; II, 113-145; III, 185-217; IV, 256-292; V, 314-352; VI, 375-411; VII, 430-470). The last three domains each contain a sequence motif resembling the cation binding site consensus sequence of D-X-D/N-X-D/N-G-X-X-D found in a number of calcium-binding proteins (Van Eldik et al., 1982). A fourth site, residues 230-238, with weak homology to this calcium binding site motif resides between repeated domains III and IV. These potential cation binding sites are in a region (residues 189-488) of the molecule that is devoid of cysteine residues.

The migration of  $\alpha_6$  in SDS-polyacrylamide gels under reducing conditions indicated that  $\alpha_6$  consisted of two poly-

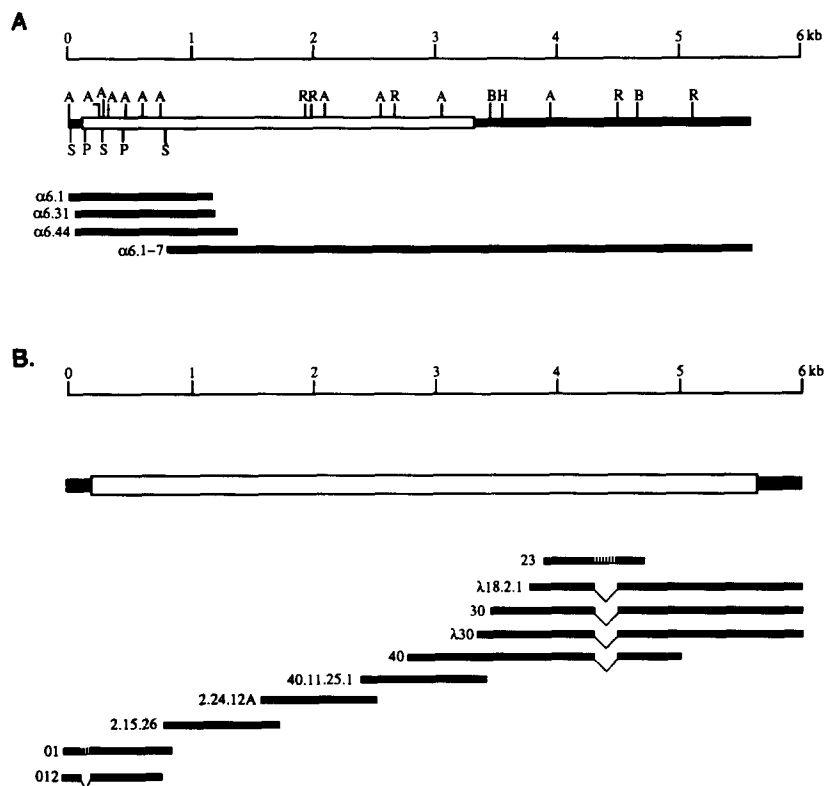


Figure 1. Restriction map of the  $\alpha_6$  (A) and  $\beta_4$  (B) cDNA clones. The ORFs for  $\alpha_6$  and  $\beta_4$  are shown as open bars. The lines indicate the size and position of the plasmid and phage cDNA clones isolated from the three cDNA libraries. Restriction sites relevant to subcloning and sequencing are Ava I (A), Bam HI (B), Pvu II (P), Eco RI (R), and Sma I (S). Representative clones out of a total of 54 are shown for  $\beta_4$ .



peptides joined by a disulfide bridge (Sonnenberg et al., 1987; Kajiji et al., 1989) similar to the integrin  $\alpha$  subunits  $\alpha_5$ ,  $\alpha_V$ ,  $\alpha_{IIb}$ , and  $\alpha_{PS2}$ . In the region of  $\alpha_6$  corresponding to this potential cleavage site (residues 899–923), there are three sets of dibasic residues. The use of two of these sites could account for the appearance of the smaller polypeptide as a doublet on SDS-polyacrylamide gels (Sonnenberg et al., 1987). This doublet may also arise from differences in glycosylation at the two sites present in the light chain. The sequence RKKRE, which most closely resembles the cleavage site of other integrin  $\alpha$  chains, is at position 899–903. Cleavage at this site would result in the formation of a heavy chain of 118,000  $M_r$  and a light chain of 24,000  $M_r$ , which are close to the 125,000  $M_r$  and 30,000  $M_r$  estimated by SDS-PAGE.

### Comparison of $\alpha_6$ with the Other Integrin $\alpha$ Subunits

Alignment with the other integrin  $\alpha$  subunits shows that  $\alpha_6$  shares several structural features with these proteins (Fig. 3). Of the 20 cysteines in the mature  $\alpha_6$  protein, 11 are in equivalent positions to those found in the other 10  $\alpha$  chains, 5 are shared with 9 of the other 10 alphas, and 2 are shared with the other 5  $\alpha$  chains which do not contain the I domain. One cysteine (residue 1039) is shared with the PS2  $\alpha$  chain, and another cysteine (residue 643) is unique to  $\alpha_6$ . Cysteine 643 is in a region of the molecule (residues 641–689) in which two gaps of about 15 and 9 amino acids were placed in the other  $\alpha$  chains in order to optimize the alignment. Three of the four putative cation binding sites of  $\alpha_6$  align with similar sites in the other  $\alpha$  subunits. A fourth weakly homologous cation binding site (residues 230–238) precedes, slightly, the cation binding sites present in the other  $\alpha$  chains which lack the I domain. The cytoplasmic domain of  $\alpha_6$  contains the sequence GFFKR which is absolutely conserved in all but the *Drosophila* PS2  $\alpha$  subunit, where the K is replaced by an N.

Some of the integrin  $\alpha$  chains contain additional polypeptide sequence.  $\alpha_2$  and the  $\alpha$  chains associated with  $\beta_2$  ( $\alpha_L$ ,  $\alpha_M$ ,  $\alpha_{P150}$ ) contain an insert domain (I domain) near the amino terminus of these molecules and the *Drosophila* PS2  $\alpha$  subunit contains an insert just preceding the transmembrane portion of this molecule.  $\alpha_6$  does not contain either of these inserts.

Based on the alignment shown in Fig. 3,  $\alpha_6$  shares 18–26% identity with the other integrin alpha subunits. The highest homology is with  $\alpha_V$ ,  $\alpha_5$ , and  $\alpha_{PS2}$  (25–26% identity) and the lowest homology is with those  $\alpha$  chains which contain the I-domain (18% identity).

### Further Evidence for the Identity of the $\alpha_6$ cDNA Clones and Association of $\alpha_6$ with Both $\beta_4$ and $\beta_1$

A rabbit antiserum (anti- $\alpha_6$  cyto) was prepared to a syn-

thetic peptide corresponding to the last 15 amino acids of the deduced  $\alpha_6$  amino acid sequence. This antiserum precipitated the  $\alpha_6\beta_4$  complex from a radiolabeled FG lysate (Fig. 4 A), indicating that the cDNA clones isolated in this study encode the  $\alpha_6$  protein. We then tested this antiserum with platelets that express  $\alpha_6\beta_1$  (Sonnenberg et al., 1987) but not  $\alpha_6\beta_4$ . Fig. 4 B shows that this antiserum precipitated the  $\alpha_6\beta_1$  complex from a radiolabeled platelet lysate. These results provide further evidence that  $\alpha_6$  forms stable heterodimers with both  $\beta_1$  and  $\beta_4$  (Hemler et al., 1989). However, they do not exclude the possibility of slight structural variations in  $\alpha_6$  which may be responsible for preferential association with either of the  $\beta$  chains.

### Isolation of cDNA Clones Encoding $\beta_4$

The cDNA for  $\beta_4$  was isolated by antibody screening of a phage expression library followed by colony hybridization of two plasmid libraries (see Materials and Methods) (Fig. 5 A). Overlapping clones covering  $\sim 5.9$  kb were sequenced (Fig. 1 B) and shown to encode  $\beta_4$  protein because: (a) they contain an ORF of 1,822 amino acids starting with a 27-residue putative signal peptide followed by a 13 amino acid sequence identical to the  $\beta_4$  protein amino terminus we previously determined (Kajiji et al., 1989); (b) their nucleotide sequence is essentially identical to that of recently reported  $\beta_4$  cDNA clones from normal epithelial cells (Suzuki and Naitoh, 1990; Hogervorst et al., 1990), with the exception of two locations described in detail below. To summarize the salient features, the mature  $\beta_4$  protein contains a 710 amino acid extracellular domain, showing obvious sequence homology to integrin  $\beta$  chains (including cysteine conservation at 47/56 positions), followed by a predicted 23 amino acid transmembrane region, and finally 1,089 amino acids that presumably represent an unusually large cytoplasmic domain. The total mass of the mature protein is 200,000 or 212,500 if all five extracytoplasmic putative N-glycosylation sites are used.

To test whether or not the large carboxyl-terminal sequence was part of the  $\beta_4$  protein in complex with  $\alpha_6$ , a native FG lysate was immunoprecipitated with the antibodies eluted from the cytoplasmic domain fusion protein (clone lam-18.2.1). These antibodies precipitated a complex of proteins with SDS-PAGE mobilities identical to those of authentic  $\alpha_6\beta_4$  heterodimers (Fig. 5 B).

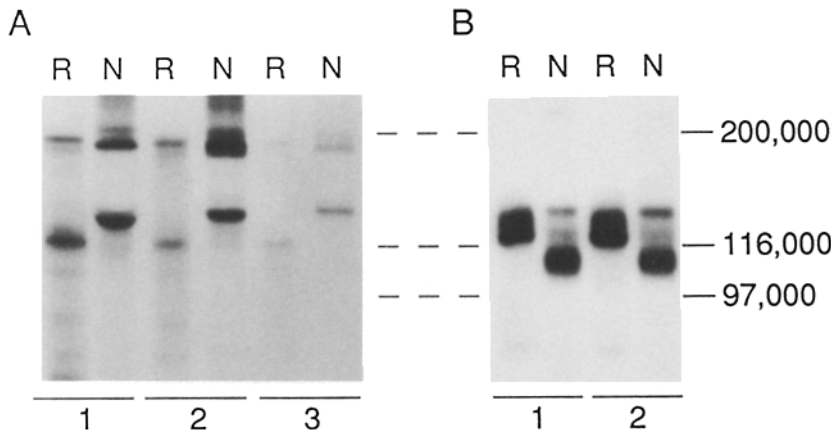
### Evidence for Possible Multiple Forms of $\beta_4$ mRNAs

Some of the cDNA clones were found to contain insertions in two locations: one insertion was identified in the 5'-untranslated region, and the other in the coding region corresponding to the cytoplasmic tail.

Thirteen independent clones, covering the 5' end of the  $\beta_4$  cDNA, were sequenced. Six of them had an insertion of 49

*Figure 2.* Nucleotide sequence and deduced amino acid sequence of the human  $\alpha_6$  subunit. The sequence corresponding with the amino-terminal sequence determined on  $\alpha_6$  protein (Kajiji et al., 1989) is underlined. The putative transmembrane region is shown by a dashed underline. The arrowhead marks the position of cleavage between the signal sequence and the mature protein. Cysteines are circled. Potential sites of N-linked glycosylation are shown in bold face. Closed boxes outline the putative cation binding domains. Dashed boxes outline dibasic residues that may represent sites of cleavage in the formation of a heavy and a light chain. The cytoplasmic sequence GFFKR, which is conserved in virtually all of the integrins  $\alpha$  chains, is denoted by a stippled dashed box. A potential polyadenylation signal is boxed. These sequence data are available from EMBL/GenBank/DBJ under accession number X53586.





**Figure 4.** Immunoprecipitation of  $\alpha_6\beta_4$  and  $\alpha_6\beta_1$  with anti- $\alpha_6$  cyto, an antibody raised to a synthetic peptide corresponding to the carboxy terminus of  $\alpha_6$ . (A) Autoradiogram of immunoprecipitations of a detergent lysate of [ $^{35}\text{S}$ ]methionine-labeled FG cells analyzed by SDS-PAGE under reducing (R) or nonreducing (N) conditions. Antibodies used were: anti- $\alpha_6$  cyto (lanes 1), antiserum 5710 (lanes 2), and the monoclonal antibody S3-41 (lanes 3), both to  $\alpha_6\beta_4$ . (B) Autoradiogram of a SDS-PAGE analysis of immunoprecipitations of  $^{125}\text{I}$  surface-labeled platelets with the monoclonal antibody to  $\alpha_6$ , GoH3 (lanes 1), and the antiserum anti- $\alpha_6$  cyto (lanes 2).

bp in the 5' untranslated sequence after position 129, 9 bp upstream of the ATG initiation codon (Fig. 6).

The second insertion sequence was found in the cytoplasmic tail where, in one out of the five clones analyzed, there was an insertion of 70 amino acids precisely in frame between threonine 1369 and glutamic acid 1440, located after bp 4294 (Fig. 6).

These extra sequences raised the possibility that multiple forms of  $\beta_4$  cDNA exist in carcinoma cells. In addition, a  $\beta_4$  cDNA from normal epithelial cells recently reported (Hogervorst et al., 1990) contains a 159-bp sequence in the cytoplasmic region not present in any of our clones and located after base pair 4,744 (Fig. 6, *arrowhead*).

To determine whether these sequences were actually present in cellular mRNAs, we amplified mRNAs from various cells using PCR. Oligonucleotide primers flanking the sites of insertion were synthesized (in one case, see Fig. 7 A, an internal primer was used) as described in Materials and Methods. PCRs using the primers flanking the 5' region insert generated products, consistent with the two types of cDNA we found, i.e., with or without the 49-bp insert (248 and 200 bp, respectively; Fig. 7 B, lanes 1 and 2), in two carcinoma cell lines tested (FG and LoVo). Of these, only the 200-bp band (i.e., without the insert) was obtained from placental mRNA (Fig. 7 B, lane 3). Unexpectedly, one band of 260 bp was seen in placental amplification products, suggesting the possibility of additional insertions in this region. The low intensity of the bands obtained from placental cDNA for the amplification of this region may be explained by low representation of 5' mRNA sequences due to degradation during mRNA extraction from tissues.

To confirm their identity, these PCR bands were subcloned and sequenced. From FG and LoVo PCRs, clones corre-

sponding to the 200- and 248-bp bands were found and shown to contain the correct nucleotide sequences. However, clones for the 260-bp band, as well as clones from placenta PCRs were not obtained. The identity of these bands deserves further study.

Using oligonucleotide primers specific to the region containing the insert at 4,294 bp (Fig. 7 B, lanes 4–6), a band of 255-bp was amplified from both LoVo and FG cDNAs but not from placenta cDNA, suggesting that mRNA containing this insert is expressed in carcinoma but not in normal cells.

PCR amplifications with primers flanking the insert at base pair 4,744 (Fig. 7 B, lanes 7–9) suggested that mRNAs, both with and without this insert, are present in placental tissue and that the form containing the insert is prevalent. Instead, LoVo and FG carcinoma cell mRNA produce only PCR bands corresponding to the insert-minus form. The identity of the 144-, 255-, and 354-bp bands was confirmed by the sequences of subclones from the respective PCRs.

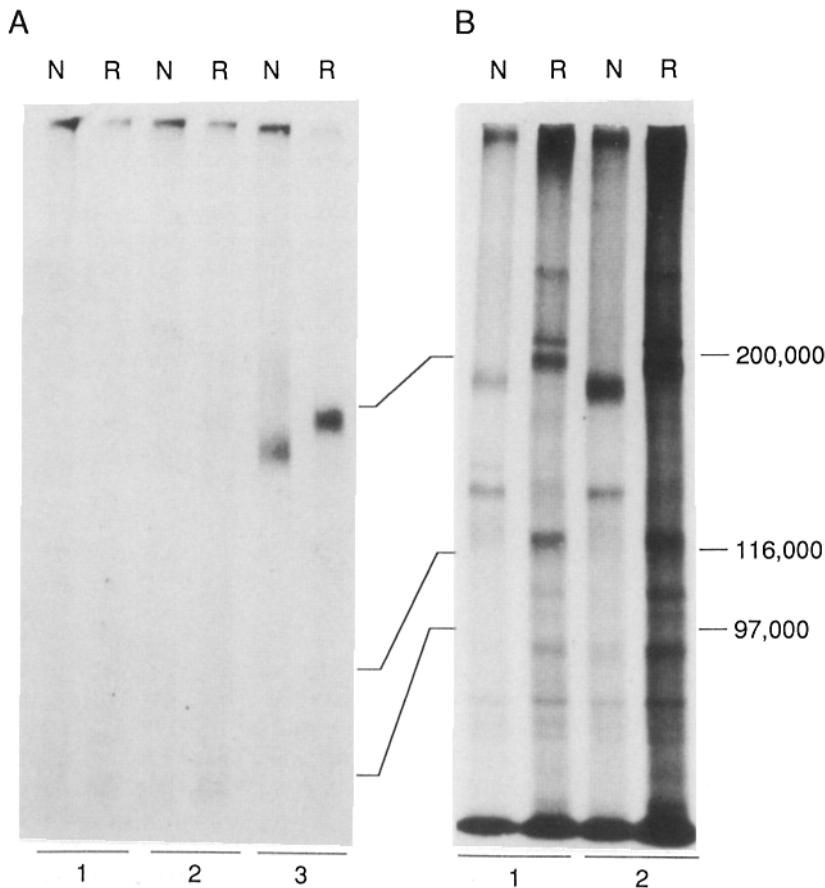
These results suggest that multiple alternatively spliced forms of  $\beta_4$  may exist in normal as well as transformed epithelial cells.

#### Cellular Distribution of $\alpha_6\beta_4$

The long cytoplasmic tail of  $\beta_4$  is suggestive of unique interactions with cytoskeletal components. To gain some information on this possibility, the cellular distribution of  $\alpha_6\beta_4$  with respect to adhesion plaques was examined by immunofluorescence studies. FG carcinoma cells were plated on Matrigel-coated glass, fixed, permeabilized, and stained by specific antibodies after 2 d of culture. The majority of the cells in these cultures had a typical epithelial appearance, forming clusters of 3–20 cells with epithelioid geometry, as

**Figure 3.** Alignment of  $\alpha_6$  deduced amino acid sequence with the other integrin  $\alpha$  subunits. The other  $\alpha$  chains are from human sources except where noted: 5 = fibronectin receptor (Argaves et al., 1987; Fitzgerald et al., 1987), V = vitronectin receptor (Suzuki et al., 1987), IIB = gpIIB (Poncz et al., 1987), 4 = VLA-4 (Takada et al., 1989), 2 = VLA-2 (Takada and Hemler, 1987), P150 (Corbi et al., 1987), M = MacI (Arnaout et al., 1988; Corbi et al., 1988), L = LFAI (Larson et al., 1989); M(M) = murine MacI (Pytela, 1988), and the position specific (PS2)  $\alpha$  chain from *Drosophila* (Bogaert et al., 1987). Initial alignment was generously provided by Dr. Robert Pytela and modified to include the  $\alpha_6$  sequence with the aid of sequence comparison programs (Devereux et al., 1984). Closed circles and open circles represent positions where a residue is conserved among all 11  $\alpha$  chains or in 10 of 11 chains, respectively. Inverted triangles denote residues found in all six (*closed*) or five or six (*open*) of the  $\alpha$  subunits which do not contain the I-domain. Potential cation binding sites are boxed with a closed line and a highly conserved cytoplasmic sequence (GFFKR) is boxed with a dashed line.





**Figure 5.** Immunoprecipitation of  $\beta_4$  with antibodies selected with the lam 18.2.1 fusion protein. (A) Antibodies eluted from plaques lam 23.1.1 (lanes 1), lam 8B (lanes 2), and lam 18.2.1 (lanes 3) were used for immunoprecipitations of a denatured [ $^{35}$ S]methionine FG lysate and analyzed by SDS-PAGE under reducing (R) or nonreducing (N) conditions. (B)  $\beta_4$  specific monoclonal antibody, AA3 (lanes 1) and antibodies eluted from plaque lam 18.2.1 (lanes 2) were used for immunoprecipitations of a nondenatured [ $^{35}$ S]methionine FG lysate and analyzed by SDS-PAGE.

evidenced by phase-contrast microscopy (Fig. 8, 1 A, 2 A, and 3 A). That these cells form adhesion plaques was shown by staining with antibodies to vinculin and talin, which can be visualized as spots on the external edges of spread cells in the periphery of the clusters (Fig. 8, 1 B and 3 B). Actin, specifically stained by fluorescent phalloidin, was also observed on the outer edges of spread cells in a pattern of spikes, and on the inside of these cells in small patches (Fig. 8, 2 B). However, no clearly recognizable actin stress fibers were seen.

In cells stained simultaneously for both talin and  $\beta_4$ , the talin staining on the outer edges was often juxtaposed to that of  $\beta_4$  (Fig. 8, 3 B and 3 C, arrows) but was not overlapping. In parts of the basal surface interior to the edge of the cells, talin was not detectable, while  $\beta_4$  was visible and appeared to be arranged in small patches and streaks (Fig. 8, 3 C, arrows). Cells double-stained with both anti-vinculin (mouse monoclonal) and anti- $\alpha_6$  cytoplasmic tail (rabbit antibodies) gave very similar patterns to the cells double stained for talin and  $\beta_4$  (not shown). These results suggest that the cellular distribution of  $\alpha_6\beta_4$  is distinct from that of adhesion plaques.

### Discussion

We have isolated cDNA clones encoding both  $\alpha$  and  $\beta$  subunits of the epithelial integrin,  $\alpha_6\beta_4$ . The  $\alpha$  subunit was sequenced from two overlapping clones encoding a protein of 1,073 amino acids. Identification of these clones as  $\alpha_6$

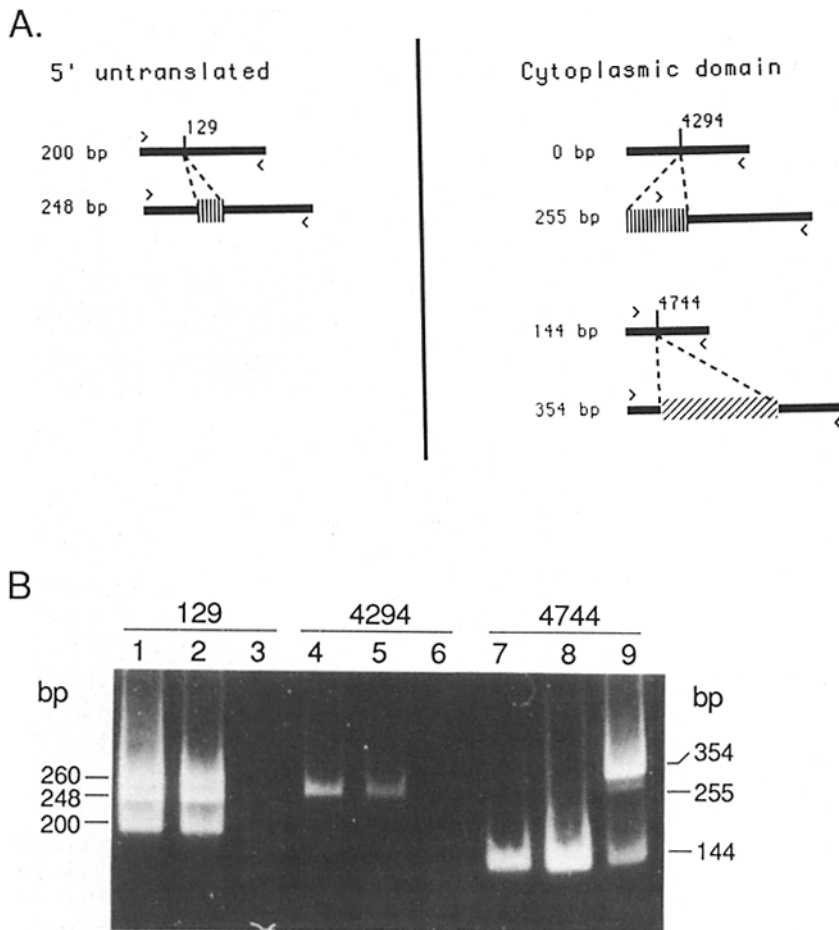
came from two pieces of evidence. First, the ORF contained a sequence that matched exactly the sequence of the amino terminus of the mature  $\alpha_6$  protein (Kajiji et al., 1989). Second, an antiserum to a synthetic peptide corresponding to the putative  $\alpha_6$  carboxy terminus in the ORF recognized the  $\alpha_6\beta_4$  complex in immunoprecipitations of radiolabeled carcinoma cell lysates.

Structurally,  $\alpha_6$  is very similar to the other integrin  $\alpha$  subunits that have been cloned to date. It shares 23–26% identity with those subunits which do not contain the I domain insert ( $\alpha_5$ ,  $\alpha_v$ ,  $\alpha_{IIb}$ ,  $\alpha_4$ , and  $\alpha_{PS2}$ ) and ~18% identity with those  $\alpha$  chains that do contain it ( $\alpha_M$ ,  $\alpha_L$ ,  $\alpha_{P150}$ , and  $\alpha_2$ ) (see alignment shown in Fig. 3).

All of the integrin  $\alpha$  proteins contain three potential cation binding sites. A fourth potential site exists in four of the  $\alpha$  chains which do not contain the I domain. In contrast,  $\alpha_6$  lacks this fourth cation binding site which makes it, along with  $\alpha_4$ , an exception to the classification of integrin  $\alpha$  subunits proposed by Takada and Hemler (1989). There is a site (residues 230–238) with weak homology to the cation binding site consensus sequence. However, this site does not align with a potential site in any of the other  $\alpha$  chains. It starts with an asparagine instead of an aspartic acid residue, and it contains a phenylalanine where an aspartic acid or asparagine residue is expected. These differences are, for the moment, of unclear significance, and await elucidation from x-ray crystallographic data.

Similar or identical forms of  $\alpha_6$  have been reported to associate with either of two integrin  $\beta$  subunits,  $\beta_1$  or  $\beta_4$





**Figure 7.** PCR products from carcinoma and placental cDNA primed with oligonucleotides for potential alternative splice sites. (A) Diagrams illustrating the location and expected size of PCR products. Solid lines represent the regions of the cDNAs being amplified. The boxes with vertical lines represent the potential inserts observed in this study, and the box with diagonal lines represents the potential insert in the  $\beta_4$  sequence reported by Hogervorst et al. (1990). Numbers to the left of each line are the size of the expected PCR product. Numbers above each line are the nucleotide positions just preceding the insert based on the numbering scheme shown in Fig. 6. Position and orientation of primers is indicated by the arrowheads. Oligonucleotides are described in Materials and Methods: 1 + 2 were used for insert 129, 3 + 4 were used for insert 4,294, and 5 + 6 were used for insert 4,744. (B) PCR products from FG cDNA (lanes 1, 4, and 7), LoVo cDNA (lanes 2, 5, and 8), and placental cDNA (lanes 3, 6, and 9) were analyzed on a polyacrylamide gel as described in Materials and Methods.

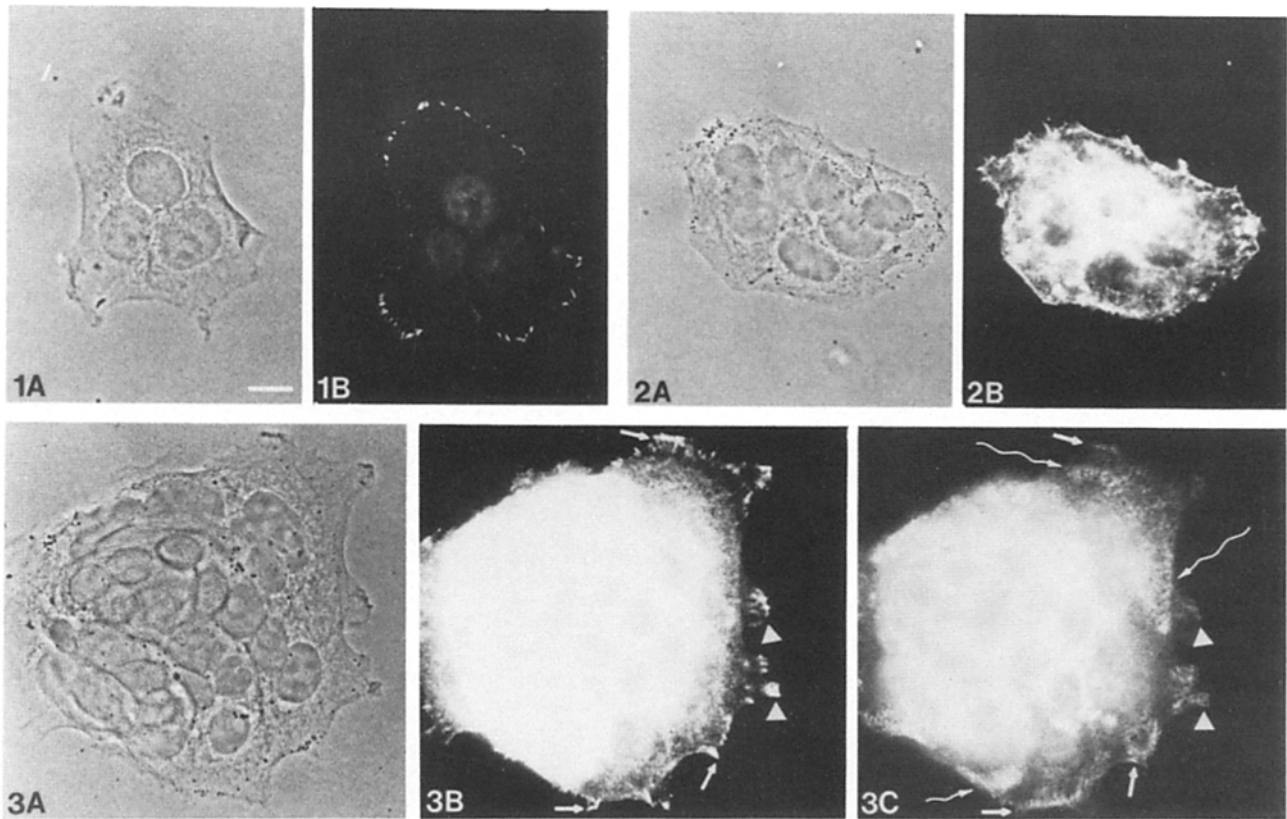
ous suggestion that the three different forms of  $\beta_4$  ( $M_r = 205,000, 180,000, \text{ and } 150,000$ , Kajiji et al., 1989) may be due to large amounts of sialic acid appears to be incorrect. Proteolytic cleavage may be a more accurate explanation for the heterogeneity in the molecular mass of  $\beta_4$  as suggested by others (Hemler et al., 1989).

The  $\beta_4$  sequences we obtained from carcinoma cells contain a few differences compared to the  $\beta_4$  cDNAs isolated from normal epithelial cells (Hogervorst et al., 1990; Suzuki and Naitoh, 1990). A 49-bp insertion located 9-bp upstream of the ATG translational start site was found in 6 of 13 cDNAs we sequenced. It is possible that this region may be of regulatory importance (e.g., for translation efficiency). Furthermore, we found clones encoding two versions of the cytoplasmic tail of  $\beta_4$ . One of these is identical to that reported by Suzuki and Naitoh (1990). The other contains an insertion of 70 amino acids after threonine 1,369. Both of our sequences are different from that reported by Hogervorst et al. (1990), which contains an insertion of 53 amino acids after histidine 1,519. Thus, it appears that the  $\beta_4$  cytoplasmic domain can exist in at least three versions.

A likely explanation for some or all of these insertions is alternative exon splicing of primary mRNA transcripts. Such mechanisms have been described in many other mRNAs (Smith et al., 1989), including the integrin subunits  $\beta_3$  (Van Kuppevelt et al., 1989) and PS2  $\alpha$  (Brown et al., 1989). Consistent with the possibility of alternative splicing, we detected PCR amplification products of the expected size using

primers located either within or adjacent to the insertions. That mature cytoplasmic mRNA, rather than immature nuclear transcripts, acted as templates in these PCR reactions is suggested by the fact that independent poly-(A) + RNA preparations yielded identical results, and that the relative ratio of the various forms remained constant within, and was characteristic of, a given cell type. For instance, the 4,744 insert was detectable in placenta but not in carcinoma cells, while the 4,294 insert had the opposite pattern (Fig. 7 B). Further experiments are, however, necessary to conclusively identify the origin of the multiple forms of the  $\beta_4$  mRNA and to gain insight into their functional significance. In particular, it is necessary to verify the existence of distinct protein forms corresponding to the three versions of the  $\beta_4$  cytoplasmic domain.

The unique cytoplasmic domain of  $\beta_4$  suggests intracellular interactions distinct from those of other integrins. When engaged with extracellular matrix ligands, several types of integrins participate in the formation of structures called adhesion plaques (or focal adhesions) characterized by the colocalization of integrin receptors with the adhesion plaque components vinculin and talin, and with the ends of actin stress fibers (Buck and Horwitz, 1987). By immunostaining, we have shown here that the  $\alpha_6\beta_4$  integrin is localized at the basal surface of adhering carcinoma cells (i.e., in areas of the plasma membrane contacting substrate). However, the distribution of  $\alpha_6\beta_4$  is distinct from that of talin, suggesting that  $\alpha_6\beta_4$  may not participate in adhesion plaque formation



**Figure 8.** Immunofluorescence staining of FG carcinoma cells. The cells were cultured on Matrigel-coated glass coverslips for 2 d, and then fixed and detergent permeabilized. (1 A) Phase contrast; (1 B) anti-vinculin antibodies. (2 A) Phase contrast; (2 B) phalloidin (actin-binding). (3 A) Phase contrast; (3 B) anti-talin antibodies; (3 C) simultaneous staining with anti- $\beta_4$  antibodies. The plane of focus was kept at the basal surface of the cells. In 3 B and 3 C areas of interest are indicated as follows: (a) wavy arrows, areas internal to the edges stained by anti- $\beta_4$  antibodies but not anti-talin antibodies; (b) straight arrows, areas on the edges of peripheral, spread cells where talin and  $\beta_4$  staining are juxtaposed but not overlapping; (c) arrowheads, areas where talin and  $\beta_4$  staining were observed by careful focussing to be distinct. See text for further explanations. Bar, 10  $\mu\text{m}$ .

in adhering carcinoma cells. It is possible that an inappropriate substrate was used in our experiments, as the ligand of  $\alpha_6\beta_4$  is not biochemically identified. However, we have made similar observations on cultured keratinocytes that can be detached by anti- $\beta_4$  antiserum (DeLuca et al., 1990). Together with the unusual  $\beta_4$  cytoplasmic tail, these data suggest that the biology of  $\alpha_6\beta_4$  may differ substantially from other well-characterized integrins.

In conclusion, while the role of  $\alpha_6\beta_4$  in epithelial cell adhesion remains to be established, the availability of  $\alpha_6$  and  $\beta_4$  cDNAs should now facilitate this task.

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**Note Added in Proof.** The sequence of  $\alpha_3$  from hamster was recently published (Tsuji, T., F. Yamamoto, Y. Miura, K. Takio, K. Tatani, S. Pawar, T. Osawa, and S. Hakomori. 1990. *J. Biol. Chem.* 265:7016–7021). Alignment with the  $\alpha_6$  amino acid sequence resulted in 37% identity, significantly higher than all other integrin  $\alpha$  chains.

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