Proteolytic Processing of Endogenous and Recombinant α4 Integrin Subunit

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Abstract. The αβ4 integrin is a receptor involved in the interaction of epithelial cells with basement membranes. This integrin is unique among the known integrins in that its β4 subunit has a large cytoplasmic domain. The function of this cytoplasmic domain is not known. In this paper we show that the β4 subunit undergoes proteolytic processing in cultured cells and provide evidence that this also happens in tissues. Immunoprecipitation experiments indicated that the cytoplasmic domain of β4 is susceptible to a calcium-dependent protease present in cellular extracts. In vitro assays with purified calpain showed that this enzyme can cleave β4 at two distinct sites in the cytoplasmic domain, generating truncated molecules of 165 and 130 kD. Immunoblotting experiments performed on cultured epithelial cells using an antibody to a peptide modeled after the COOH-terminus of the β4 subunit showed 70-kD fragments and several fragments of molecular masses between 185 and 115 kD. Similar fragments were detected in CHO cells transfected with the full-length β4 cDNA, but not in control transfected cells or in cells transfected with a mutant cDNA lacking the epitope of the cytoplasmic peptide antibody. The sizes of the fragments indicated that both the intracellular and extracellular domains of β4 are proteolytically processed. To examine the processing of the β4 subunit in epithelial tissues in vivo, human skin frozen sections were stained with antibodies to the ectodomain or the cytoplasmic domain of β4. The distinct staining patterns obtained with the two types of antibodies provided evidence that β4 is proteolytically processed in vivo in skin. Analogous experiments performed on sections of the cornea suggested that β4 is not proteolytically processed at a detectable level in this tissue. Thus, cleavage of the β4 subunit occurs in a tissue-specific fashion. These results suggest a potential mechanism of modulating the activities of the αβ4 integrin.

The αβ4 integrin is expressed by epithelial and neuroepithelial cells (22). In epithelial tissues, this integrin is restricted to the basal portion of those cells which contact the basement membrane, suggesting a role in adhesion to basement membranes (4, 12, 22, 24, 25). Its ligand may be laminin or a laminin isoform (15, 23). The primary sequences of both the α and the β subunits have been recently determined (10, 27, 28). The β subunit is unique among integrin β subunits because of the large size of its cytoplasmic domain. This part of the molecule, which measures over 100 kD, contains two pairs of type III fibronectin repeats interrupted by a sequence which shows variations, probably as a result of alternative splicing of mRNA (10, 27, 28). Type III fibronectin repeats are commonly found in extracellular matrix molecules and in the external portion of many adhesion receptors (16). In addition to the intracytoplasmic portion of β4, two other known intracellular molecules with such repeats are twitchin and titin, two related proteins of the myofibrillar cytoskeleton (1). It is possible that the β4 gene has originated by the junction of exons encoding an ancestral integrin with exons of a cytoskeletal molecule.

The β4 subunit is identical to the so-called Tumor Specific Protein 180 (TSP180), a molecule whose expression is increased in invasive carcinomas relative to benign adenomas and normal tissues (13). The β subunit might therefore be regulated by transformation differently from the αβ3 fibronectin receptor, whose expression is decreased in fibroblasts transformed by viril oncogenes (17).

To examine the function of the αβ4 integrin, we have begun gene transfer experiments. During the initial phase of these studies, we observed that the β subunit undergoes an in vivo proteolytic processing affecting both the extracellular and the intracellular domains of the molecule. This processing, which is also observed in CHO cells transfected with a β expression construct, is specific to β4 and does not occur with other integrin β subunits.

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Materials and Methods

Peptides, Antibodies, and Cell Lines

Peptides were synthesized by using a peptide synthesizer (Applied Bio-systems, Foster City, CA) and solid-phase t-boc chemistry. After cleavage and deprotection with trifluoroethanesulfonic acid, the peptides were purified by HPLC chromatography.

The α8 subunit–specific rabbit polyclonal antibody was elicited by immunization with a 29-mer synthetic peptide (NKHDYDATYHKAEICTRHQPVK) reproducing the COOH-terminal 29-amino-acid portion of the human α8 subunit (28). The β4 subunit–specific rabbit polyclonal antibody was raised against the synthetic peptide CTRHVTQEFVSRTLTGSTLTHMDQDFQFG (31-mer). This peptide is modeled after the COOH-terminal segment of the large cytoplasmic domain of the human β subunit (10, 27). Both peptides were conjugated to keyhole limpet hemocyanin for immunization. In order to obtain peptide-specific antibodies, the β4 cytoplasmic peptide serum was affinity purified on a column made with the peptide used for immunization. To this end, the peptide was crosslinked to BSA at a molar ratio of 50:1 using glutaraldehyde. The BSA-peptide conjugate was then covalently linked to CNBr-activated Sepharose. This procedure was used because it allows for efficient exposure of the peptide to the fluid phase of chromatography. The α8 and β4 subunit–specific cytoplasmic peptide antibodies were previously described (6). The 3E1 mAb (9) recognizes the extracellular portion of the β4 subunit, as demonstrated by staining of intact live cells. The 341-IAA rat mAb to mouse β3 (13) was provided by Dr. S. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). The 341-OP3 rat mAb to α8 (23) was provided by Dr. A. Sonnenberg (Central Laboratory of the Netherlands Red Cross and University of Amsterdam, Amsterdam, The Netherlands).

The SW480 human colon carcinoma cells and the A431 epidermoid carcinoma cells were cultured in DME with 10% FBS. Primary human foreskin keratinocytes were maintained in serum-free keratinocyte growth medium supplemented with insulin, EGF, hydrocortisone, and bovine pituitary extract (Clonetics Corp., San Diego, CA). The dihydrofurotase reductase (dfhr)-negative CHO-DG44 cells (29) were grown in nucleoside-free, a- minus MEM supplemented with 10% FBS, hypoxanthine, and thymidine. Transfected CHO-DG44 cells were cultured in α-minus MEM supplemented with 10% dialyzed FBS.

Expression Constructs and Transfection

A full-length cDNA clone for the human β3 subunit was assembled in the cytomegalovirus promoter-based expression vector PRC-CMV (Innvitro Corp., San Diego, CA). The two overlapping partial clones 6αA-1 (5.6 kb) (27) and 6αA-E2 (1.6 kb), which cover the entire coding sequence of β3, were subcloned into the EcoRI site of pBSK-1 and pBSK-E2. The 0.9-kb HindIII-XbaI fragment of pBSK-E2 was then ligated in pRC-CMV. The resulting plasmid was digested with BspM2 and XbaI and ligated to the 4.9-kb BspM2-XbaI fragment of pBSK-1. The resulting expression construct, encoding a full-length β3 subunit, was designated pCMV-β3. An expression vector encoding a truncated tailless β3 subunit was created from pCMV-β3 by engineering a stop codon at amino acid position 855. The pCMV-β3 vector was digested with Accl and, after partial filling in with C+T, cut with BspEI. The resulting 6.2-kb fragment was ligated to the 1.9-kb BspEI-Accl fragment of pCMV-βα. The procedure of adjoining the Accl end at position 2687 with the partially filled XbaI end generated a stop codon at amino acid position 855. Correctness of this construct, termed pCMV-β3Δcyto, was verified by sequencing. Sequencing was performed by the dideoxy chain termination method using Sequenase (United States Biochemical Corp., Cleveland, OH). The restriction enzyme were from New England Biolabs (Beverly, MA), Stratagene (La Jolla, CA), and Boehringer Mannheim Biochemicals (Indianapolis, IN). T4 DNA ligase, calf intestinal phosphatase, the large fragment of Klenow polymerase, and nucleotides were from Boehringer Mannheim Biochemicals.

CHO-DG44 cells were transfected with 10 μg of either pCMV-β4 or pCMV-β4Δcyto. Cotransfection of 1 μg of pSV2-dfhr (26) provided the selection marker. The dfhr-positive cells were selected in nucleoside-free medium and pooled. Clones with high level expression of the transfected β4 molecules were selected by three cycles of fluorescence-activated cell sorting with the anti-human β4 3E1 mAb. Clonal cell lines were then obtained by limiting dilution.

Control cell lines were generated by transfection of pSV2-dfhr alone and subsequent selection in nucleoside-free medium. Two independent control clones, C1 and C5, the pCMV-β4 transfected clone B10, and the pCMV-β4Δcyto transfected clone D2 were used throughout this study.

Immunoprecipitation Analyses

For immunoprecipitation, cells were labeled in suspension with 125I by the lactoperoxidase-H2O2 method as previously described (18) or metabolically labeled with 50 μCi/ml of [35S]methionine (>800 Ci/mmol; New England Nuclear) for 6 h. After washing, the cells were extracted with lysis buffer containing 0.5% Triton X-100, 30 mM Tris (pH 7.5), 150 mM NaCl, 0.01% aprotinin (Sigma Chemical Co., St. Louis, MO), 4 μg/ml leupeptin (Sigma Chemical Co.), and either 1 mM CaCl2 or 10 mM ethylenediaminetetraacetate (EDTA). For experiments of in vitro cleavage of α8β4 by calpain, the protease inhibitors in the lysis buffer were replaced with 10 mM EDTA, or 20 μM CaCl2, or 20 μM CaCl2 and 100 μM N-acetyl-leucyl-leucyl-norleucinal (Calpain Inhibitor I; Boehringer Mannheim Biochemicals). Immunoprecipitation analyses were performed as previously described (6). All the immunoprecipitations were analyzed by SDS-PAGE (14) and autoradiography with X-Omat AR films (Eastman Kodak Co., Rochester, NY).

In Vitro Digestion with Calpain

To obtain radiolabeled intact β3 subunit, the α8β3 integrin was immunoprecipitated from [35S]methionine–labeled SW480 cells. The extraction buffer contained 0.5% Triton X-100, 50 mM Tris (pH 7.5), 150 mM NaCl, 0.01% aprotinin (Sigma Chemical Co.), 4 μg/ml leupeptin A (Sigma Chemical Co.), 10 μg/ml leupeptin (Sigma Chemical Co.), 10 mM EDTA, and 100 μM Calpain Inhibitor I. The sample was precipitated with the 3E1 mAb and Sepharose-conjugated anti–mouse IgG. The Sepharose-bound material was washed with lysis buffer without protease inhibitors and equilibrated in calpain digestion buffer (10 mM Tris, pH 7.5, 25 mM NaCl, 1 mM CaCl2). The Sepharose suspension was then divided into 50-μl aliquots. Each aliquot was incubated with the indicated concentrations of purified 80-kD catalytic subunit of calpain (from rabbit skeletal muscle; Sigma Chemical Co.) for 15 min at room temperature. The samples were boiled in SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography.

Immunoblotting

Confluent cell monolayers were extracted at 95°C with SDS-PAGE sample buffer supplemented with 10 mM EDTA. Samples containing 100 μg total proteins were separated by SDS-PAGE and subjected to immunoblotting according to previously published procedures (6). The affinity-purified cytoplasmic peptide antibodies were used at 5 μg/ml. Competitive inhibition was effected by incubation of the peptide antibodies (5 μg/ml) with excess of the corresponding peptide (100 μg/ml). Specificity controls were obtained by incubating the β4 cytoplasmic peptide antibodies (5 μg/ml) with a β3 cytoplasmic peptide (100 μg/ml).

Immunohistochemistry

Human skin biopsies, mouse skin samples, and human corns, obtained from the National Direct Research Interchange (Philadelphia, PA), were frozen and embedded in OCT medium. Cryostat sections of 6 μm thickness were fixed with 3.7% paraformaldehyde in PBS or left unfixed. In some experiments, the paraformaldehyde-fixed tissue sections were permeabilized with 0.5% Triton X-100 in PBS before fixation. Staining was performed using the hybridoid culture supernatants undiluted, the ascites diluted at 1:1,000, and the affinity-purified cytoplasmic peptide antibodies at 5 μg/ml. Peptide competitions were done as described for immunoblotting. After incubation with the primary antibodies, the sections were incubated with biotinylated secondary antibodies followed by a preformed avidin-biotinylated HRP complex (Vectastain ABC; Vector Laboratories, Burlingame, CA), with FITC-conjugated donkey anti–rabbit IgG or with TRITC-conjugated donkey anti-mouse IgG.

Results

Calcium-dependent Proteolysis of β3

To examine the expression of the α8β3 integrin at the sur-
face of epithelial cells, we performed immunoprecipitation experiments with the mAb 3El that recognizes the extracellular portion of the β4 subunit. SW480 colon carcinoma cells were iodinated at the cell surface and extracted in the presence of calcium or EDTA. In addition to the α6 and β4 subunits, the 3El antibody precipitated two polypeptides with apparent molecular masses of 165 and 130 kD (Fig. 1). Comparing immunoprecipitates from calcium- and EDTA-containing extracts showed that the abundance of the 165- and 130-kD polypeptides relative to the 200-kD α4 subunit was higher in extracts containing calcium (Fig. 1, compare lanes a and b, and c and d). These results suggested that the β4 subunit can be cleaved in vitro, presumably as a result of a calcium-dependent process. In agreement with our findings, previous results have demonstrated β4-related polypeptides of molecular masses similar to the 165- and 130-kD molecules described here (8). In our experiments, EDTA did not prevent completely the appearance of these proteolytic fragments (Fig. 1, lanes a and c). We hypothesized that the fragments detected in EDTA extracts could reflect an in vivo physiological processing of the β4 subunit.

To confirm and extend these results, we performed immunoprecipitation experiments with cells labeled either metabolically with [35S]methionine or at the cell surface with 125I. SW480 cells were extracted in a detergent buffer including EDTA and other inhibitors of calcium-activated proteases. As shown in Fig. 2, only α6 and the intact form of β4 could be detected in metabolically labeled cells, indicating that the β4 subunit is stable during the initial stages of its biosynthesis and that our extraction conditions effectively prevent its in vitro proteolysis (Fig. 2, lane b). In contrast, the 165- and 130-kD proteolytic products of β4 could be detected in immunoprecipitates from surface-iodinated cells extracted and processed under identical conditions (Fig. 2, lane c). We therefore concluded that a fraction of the β4 molecules expressed in culture by SW480 cells undergoes proteolytic cleavage. As shown below, products of the proteolytic processing can also be detected in intact cells without iodination.

**Cleavage of the Cytoplasmic Domain of β4 by Calpain**

We next set out to determine whether the β4 subunit was susceptible to proteolytic cleavage by one of the isoforms of the calcium-activated enzyme calpain. SW480 cells were labeled metabolically with [35S]methionine and then extracted under different conditions: in the absence of calcium, in the presence of 20 μM calcium, or in the presence of calcium and the synthetic peptide inhibitor of calpain, N-acetyl-leucyl-leucyl-norleucinal. The extracts were immunoprecipitated either with the mAb 3El or with affinity-purified antibodies to a synthetic peptide modeled after the COOH-terminal 31-amino acid segment of the cytoplasmic domain of β4 (Fig. 3A). The experiment showed that micromolar amounts of calcium are sufficient to activate the proteolytic activity present in cellular extracts (Fig. 3, compare lanes a and c) and that this calcium-dependent proteolysis is effectively inhibited by the peptide inhibitor of calpain (Fig. 3, compare lanes c and e). The results suggested that β4 can be proteolytically cleaved in vitro by the type I isoform of calpain. Since the 165- and 130-kD proteolytic fragments of β4 were not recognized by the cytoplasmic peptide antibodies (Fig. 3, lane d), we concluded that they were the result of cleavage within the cytoplasmic portion of the molecule.

To investigate directly the susceptibility of the β4 subunit to calpain, we incubated the immunoisolated intact α6β4 integrin with different amounts of the purified enzyme. The experiment showed that calpain is able to cleave β4 in a dose-dependent way generating fragments of molecular weights...
similar to that of those generated in cellular extracts (Fig. 3 B). The pattern of digestion of the \( \beta_4 \) subunit induced by calpain was not entirely identical to that observed upon addition of calcium to cellular extracts, perhaps because in the former assay the substrate was purified, while in the latter it was not. However, the calpain-generated fragments were similar to the fragments seen in the cell extracts in that they also could not be precipitated with the cytoplasmic peptide antibody (not shown). These results show that the cytoplasmic domain of \( \beta_4 \) can be cleaved by calpain and that the resulting fragments are similar to the endogenous cleavage products.

**Proteolytic Digestion of \( \beta_4 \) in Cultured Cells**

To extend the analysis of the proteolytic processing of \( \beta_4 \), we performed immunoblotting experiments on extracts of various epithelial cell lines. Cell monolayers were extracted in SDS-PAGE sample buffer at 95°C to prevent postlytic in vitro degradation. After electrophoresis and blotting, the extracts were probed with affinity-purified antibodies to the cytoplasmic peptide of \( \beta_4 \) (Fig. 4). The antibodies reacted with two different sets of bands: a doublet at \( \sim 70 \) kD and several other bands with molecular masses between 185 and 115 kD (Fig. 4 A). The reaction was specific, because it was
inhibited by the peptide used for immunization (Fig. 4 B), but not by an irrelevant peptide. A faint 35-kD band was observed in some immunoblotting experiments. Under the same conditions and with similar cytoplasmic peptide antibodies, we could not detect any proteolytic fragment of the β1 (Fig. 4 C) or β, subunit. The 70-kD fragments have a size compatible with their being generated by the intracellular proteolytic activity of calpain. The larger fragments, however, can only originate from the digestion of the extracellular portion of the molecule. The results collectively indicated that in cell culture a subset of β4 molecules undergoes a complex pattern of proteolytic degradation involving possibly calpain, but also extracellular proteases.

We next analyzed β4 processing in CHO cell lines expressing recombinant β4 molecules from cDNA. The CHO cells, which, as shown below, express an endogenous α6β, complex and no β, subunit, were transfected with the expression construct pCMV-βdcyto encoding the full-length β4 subunit, or with the plasmid pCMV-βdACYTO encoding a truncated β4 subunit lacking most of the cytoplasmic domain. Clones with high-level expression of the recombinant subunits were selected by FACS and further analyzed by surface iodination and immunoprecipitation. Fig. 5 A shows the results obtained with representative clones. C1, a clone transfected with the selection marker plasmid alone, similarly to the parental cells, expressed an endogenous α6β1 integrin, but no β4 subunit. B10, a clone transfected with the full-length β4 cDNA, expressed an α6β1 complex recognized by both the 3EI mAb and a cytoplasmic peptide antiserum to α6. The recombinant human β4 subunit therefore associated with the endogenous α6 subunit and it was correctly exported to the cell surface. D2, a clone transfected with the plasmid pCMV-βdACYTO, expressed at the cell surface a complex of α6 with the 95-kD truncated β4 subunit. Thus, the truncated β4 subunit was also correctly expressed at the cell surface.

Immunodepletion experiments showed that expression of the β4 subunit in association with α6 was greatly reduced in the clones expressing recombinant β4 molecules (Fig. 5 A). The reduction of α6β1 was proportional to the level of expression of the transfected β4 cDNA in different clones (not shown). These results suggest that the α6 subunit has a higher affinity for β4 than for β1. To address this point directly, we examined the transfected cells for the synthesis rate of β1, β4, and the two main α subunits expressed by CHO cells, α6 and α5. As shown in Fig. 5 B, clone B10 synthesizes the recombinant β4 subunit at a rate lower than that of the endogenous β1. The same clone expresses at the cell surface high levels of the α6β1 integrin, but the amount of α6β1 is reduced relative to the control clone. We conclude therefore that α6 combines more efficiently with β4 than with β1.

The experiments of surface iodination and immunoprecipitation shown in Fig. 5 A suggested that the recombinant β4 subunit expressed in CHO cells undergoes a proteolytic processing similar to that of the endogenous β4 molecule in epithelial cells. To examine this question, control clones and clones transfected with the full-length or the truncated β4 cDNA were extracted in SDS-PAGE sample buffer at 95°C and subjected to immunoblotting (Fig. 6). In cells expressing the full-length β4 cDNA, the β4 cytoplasmic peptide antibodies reacted with the intact recombinant β4 molecule, with a number of high molecular mass fragments, and with a doublet at ~70 kD (Fig. 6, A, lane b). No polypeptide was recognized by these antibodies in extracts of control cells.
Its proteolytic fragments are generated by the 3El mAb is due to a limited accessibility of its epitope on the lateral and apical surfaces of the basal cells, because an identical pattern was obtained with antibodies (3El, GOH3, 346-11A) recognizing epitopes in the ectodomain of both the \( \alpha_4 \) and \( \beta_4 \) subunits and by using different fixation and permeabilization procedures. Moreover, published studies show that antibodies to the \( \alpha_4 \) and \( \alpha_6 \) subunits stain all surfaces of the basal layer keratinocytes, indicating access of antibodies to every aspect of these cells (see, e.g., ref. 2). The data therefore suggest that \( \beta_4 \) molecules bearing the COOH-terminal epitope of the peptide antibody, but lacking the extracellular epitope of the 3El antibody, exist within the cytoplasm of basal keratinocytes. Therefore, these results suggest that the proteolytic processing of \( \beta_4 \) observed in cultured cells also occurs in vivo in the skin.

We next compared another stratified squamous epithelium, the cornea, to the skin. In the cornea, both the 3El mAbs and the COOH-terminal peptide antibody generated an identical basement membrane–like staining pattern (Fig. 8). Some staining was seen around the corneal cells in the upper layers, but this was also similar for both types of antibodies. These results suggest that proteolysis of \( \beta_4 \) does not occur in the cornea. Thus, cleavage of \( \beta_4 \) may be regulated in vivo in a tissue-specific fashion.

**Discussion**

We show that the \( \beta_4 \) subunit undergoes a complex proteolytic processing affecting both the extracellular and intracellular domains of the molecule. The \( \alpha_6 \beta_4 \) integrin is structurally different from other integrins because of the large cytoplasmic domain of the \( \beta_4 \) subunit (10, 27). In stratified squamous epithelia, including skin, tongue, and cornea, the receptor is concentrated within the hemidesmosomes (11, 24, 25). Thus, in contrast to other integrins, \( \alpha_6 \beta_4 \) is not likely to connect to the actin filament system. Instead, it may interact, directly or indirectly, with the keratin filament system. Such an interaction may contribute to the maintenance of cell surface polarity and to the stability of the epithelial sheet. In the stratified squamous epithelia, the receptor is expressed exclusively by the cells of the basal layer (2, 4, 12, 24). Thus, expression of \( \alpha_6 \beta_4 \) is rapidly turned off at the onset of cell differentiation and movement to the upper cell layers (7).

Our immunoblotting experiments with cytoplasmic peptide antibodies revealed two sets of \( \beta_4 \) fragments: there were several fragments with sizes between 185 and 115 kD, and two fragments of \(~70 \) kD. The sizes, immunoreactivities, and heterogeneity of the larger fragments suggest that they are products of one or more extracellular proteases. The two 70-kD fragments, which represent large segments from the cytoplasmic domain of \( \beta_4 \), may be produced as a result of digestion by one of the isoforms of the calcium-activated protease calpain. We found that the cleavage of the \( \beta_4 \) cytoplasmic domain in cell extracts was due to a calcium-activated proteolytic activity and that the cleavage could be blocked by a specific peptide inhibitor of calpain. Furthermore, purified calpain cleaved the cytoplasmic segment of \( \beta_4 \) at two places. One of the cleavage regions is \(~70 \) kD from the COOH-terminus of the polypeptide, because a 130-kD fragment lacking the COOH-terminal antigenic determinant was generated. In agreement with the existence of such a cleavage, we detected two 70-kD fragments that were

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**Figure 6.** Immunoblotting of \( \beta_4 \) fragments in CHO cells expressing recombinant \( \beta_4 \) molecules. The CHO clones C1, transfected with the selection marker plasmid alone (a), B10, expressing the full-length \( \beta_4 \) cDNA (b), and D2, expressing the truncated \( \beta_4 \) cDNA that lacks the epitope of the \( \beta_4 \) cytoplasmic peptide antibodies (c), were extracted in SDS-PAGE sample buffer at 95°C. Samples containing 150 \( \mu \)g of total proteins were separated by SDS-PAGE under nonreducing conditions and transferred to nitrocellulose. Identical filters were probed with affinity-purified antibodies to the COOH-terminal peptide of \( \beta_4 \), in the absence (A) or in the presence of the peptide used for immunization (B), and with affinity-purified antibodies to the cytoplasmic peptide of \( \beta_4 \) (C).
recognized in immunoblotting by the cytoplasmic peptide antibody. These fragments differ only slightly in size, possibly reflecting the existence of two closely spaced calpain cleavage sites.

Inspection of the amino acid sequence of the cytoplasmic domain of $\beta_4$ reveals several potential cleavage sites for calpain. Cleavage at either RI289-E1290 or RI355-S1356 would generate fragments of 130 and 70 kD, while cleavage at RI529-V1530 would give 165- and 35-kD fragments. Molecules susceptible to proteolysis by calpain generally contain at least one region enriched in proline (P), glutamic acid (E), aspartic acid (D), serine (S), and threonine (T) (PEST region) (30). Such regions, which are typically located at some distance from the cleavage site, are thought to be able to bind calcium ions and facilitate the interaction of the enzyme with its substrate. We have searched the amino acid sequence of $\beta_4$ for PEST regions with the PC/GENE protein sequence analysis software. We found that at least two such regions are present in the cytoplasmic domain of $\beta_4$ (position 1685-1696 with PEST score of $-0.15$, and position 1554-1592 with PEST score of $-1.6$).

Our results show that a portion of the total $\beta_4$ in cultured cells is comprised of molecules that apparently lack a large segment of the extracellular domain. The portion lacking in these fragments includes the region that is thought to contribute to the ligand binding site (5). In addition, the prominence of the 70-kD fragments that include the COOH-terminus of $\beta_4$ suggests that a portion of $\beta_4$ molecules both in vitro and in vivo lacks a major part of the cytoplasmic domain. Proteolytic cleavage of $\beta_4$ could therefore alter both the ligand binding activity and the cytoskeletal connections of $\alpha_6\beta_4$.

We have also analyzed CHO clones expressing human $\beta_4$ subunits from cDNA. The results indicated that a fraction of the recombinant $\beta_4$ subunit expressed by these cells undergoes a proteolytic processing similar to that observed with cultured epithelial cells. The gene transfer experiments also established that the $\alpha_6$ subunit has a higher affinity for $\beta_4$ than for $\beta_1$. This latter finding provides a molecular basis for previous observations indicating that, in cells expressing $\alpha_6$, $\beta_4$, and $\beta_1$ subunits, $\alpha_6\beta_4$ generally predominates over that of $\alpha_6\beta_1$ (4, 15, 23, 25).
Figure 8. Immunofluorescence localization of $\beta_4$ in the skin and in the cornea. Unfixed frozen sections of human skin (A and B) or human cornea (C and D) were subjected to double labeling with the $\beta_4$ mAb and the affinity-purified antibodies to the COOH-terminal peptide of $\beta_4$. TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG were used to detect, respectively, the bound monoclonal (A and C) and rabbit peptide antibodies (B and D). In the skin, the staining at the basement membrane appears more intense than in the cornea. The basal aspect of the basal cell membrane in skin is undulating and thus appears thicker than a similar region of the basal cells of the corneal epithelium which is flat. Note that the COOH-terminal peptide antibodies intensely stain the cytoplasm of the basal cells of the skin (B) but not of the cornea (D).

While only a fraction of $\beta_4$ molecules underwent proteolytic processing in the cultured cells, the immunohistochemistry results suggest that in vivo in the skin a larger fraction of the $\beta_4$ molecules is proteolytically processed. In fact, staining of skin epithelium showed that the COOH-terminal cytoplasmic domain epitope was homogeneously distributed in the cytoplasm, while the ectodomain epitope was selectively localized at the basal cell surface. These data suggest that factors operating in vivo in the skin contribute to an accelerated rate of processing of $\beta_4$ in this tissue.

It is possible that proteolysis of this basement membrane receptor is related to the remodeling of adhesive and cytoskeletal structures which occurs in the skin, when the basal cells detach from the basement membrane at the onset of differentiation. This interpretation is consistent with the apparent lack of $\beta_4$ processing in the cornea, another stratified epithelium. In contrast to those of skin, the stem cells of cornea reside in the limbus, a transitional zone between the epithelium and the conjunctiva, and differentiate while moving centripetally along the basement membrane (3, 20). Thus, differentiating corneal keratinocytes maintain a more prolonged interaction with the basement membrane. Further experiments will be needed to examine this hypothesis. Taken together, our results indicate that the $\alpha_4\beta_4$ receptor, unlike other integrins, undergoes regulated proteolytic processing in vivo.

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