Cloning and Characterization of a Novel Integrin β3 Subunit*

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We have identified a novel integrin β3 subunit, termed β3C, from a human osteoclast cDNA library. The COOH-terminal sequence and 3′-untranslated region of the β3C subunit differs from the previously reported β3A (platelet) and β3B (placenta) sequences, while the regions coding for the transmembrane and extracellular domains are identical. The β3C cytoplasmic domain contains 37 amino acids, the last 17 of which are encoded by a novel exon located about 6 kilobase pairs downstream of exon 14 of the β3A gene. HEK 293 cells were stably co-transfected with αv and either β3C (HEKβ3C) or β3A (HEKβ3A). The viability of HEKβ3A cells was lower than that of HEKβ3C cells, and HEKβ3C cells in culture grew as clusters rather than as a monolayer. The novel cytoplasmic domain did not affect receptor binding affinity; both αvβ3A and αvβ3C isoforms exhibited high affinity binding to 125I-echistatin and cyclic and linear RGD peptides. However, in contrast to HEKβ3A, HEKβ3C cells failed to adhere to osteopontin, an αvβ3 matrix protein. The data provide further support for the key role of the cytoplasmic domain of the β3 integrin in cell adhesion and suggest a potential role for the β3C integrin subunit in modulating cell-matrix interactions.

Integrins are a family of transmembrane receptors that mediate cell-cell and cell-extracellular matrix interactions (1). They are heterodimers composed of noncovalently associated α and β subunits. Each subunit has a large extracellular domain, a single transmembrane region, and a short cytoplasmic domain (2, 3). There are at least 9 β subunits and 15 α subunits that associate in various combinations to generate integrins of diverse ligand binding specificity. The extracellular domains of many of the integrins bind the Arg-Gly-Asp (RGD) sequence in several extracellular matrix proteins, while the cytoplasmic tail interacts with cytoskeletal proteins and generates signals important for cell adhesion, cell motility, apoptosis, and specific gene regulation (4, 5).

One member of the family, αvβ3, mediates cell adhesion to a wide spectrum of extracellular matrix proteins, including vitronectin, von Willebrand factor, fibrinogen, thrombospondin, fibronectin, and osteopontin (6). αvβ3 is expressed on osteoclasts, platelets, melanoma, and endothelial and smooth muscle cells (7). Osteoclasts are mobile, multinucleate cells within bone that are responsible for bone resorption (8, 9). It has been proposed that αvβ3 is present on the osteoclasts mediates attachment of osteoclasts to the bone matrix. This attachment is a prerequisite for osteoclast polarization, organization of the ruffled border, and the eventual resorption of bone. Monoclonal antibodies to αvβ3 have been shown to inhibit the osteoclast-mediated bone resorption in vitro (10). An RGD containing snake venom protein, echistatin, binds αvβ3 with high affinity and inhibits bone resorption in an organ culture system (11) and in a thyro-parathyroidecortomized rat model (12). These observations suggest that αvβ3 plays an important role in modulating osteoclast function.

Several lines of evidence suggest that there may be heterogeneity in αvβ3 obtained from different tissue sources. First, an alternative form of the β3 subunit with a variant cytoplasmic domain has been identified from a human placental library (13). This variant form is generated by alternate mRNA splicing. Second, a monoclonal antibody, 10C4.1.3, which recognizes a unique epitope on αvβ3, exhibits a very narrow tissue distribution with high level expression restricted to osteoclasts (14). These results suggest that epitopic variation may exist in αvβ3 expressed on the osteoclast compared with that expressed on other tissues. Third, a recent study reported that the potency for a series of αvβ3 antagonists differed between their inhibition of bone resorption and endothelial cell adhesion on vitronectin (15), suggesting that the αvβ3 on osteoclasts may have a distinct ligand specificity.

To examine whether osteoclasts express a distinct form of αvβ3, we isolated and characterized αv and β3 cDNA clones from a human osteoclast-enriched osteoclastoma library. The αv cDNA clone was identical to the αv CDNAs obtained from endothelial and fibroblasts cells (16, 17). On the other hand, the β3 subunit existed in two forms: the previously characterized β3A form, which has a COOH-terminal sequence of 21 amino acids, and a novel β3C form with a unique 17-amino acid COOH-terminal sequence. In this report we describe the cloning and characterization of this novel β3C subunit.

MATERIALS AND METHODS

Construction and Screening of a Human Osteoclastoma cDNA Library—Human osteoclasts were isolated from osteoclastoma tissue as described previously (18). Briefly, osteoclasts were enriched from an osteoclastoma-derived cell suspension using a murine monoclonal antibody specific for the β3 chain of the vitronectin receptor (C22; Ref. 19), and magnetic beads (Dynal Inc, Great Neck, NY) coated with an anti-murine antibody. After 3 days in culture, osteoclast mRNA was extracted using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). A directional cDNA library was prepared from this mRNA (Stratagene, La Jolla, CA) in the Uni-ZAP XR vector. The average insert size of the library was greater than 1 kb.

To screen the library, αv and β3 CDNAs (3.2 and 2.4 kb in length, respectively) were generated from published sequences (20, 21) by RT-

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1 For the sake of uniformity with other β3 variants, we refer to this novel β3 subunit as β3c, to distinguish it from the β3A (platelet/endothelial cell) and β3B (placenta) subunits.
PCR² using human placental poly(A)⁺ RNA as a template. The identities of the cDNA sequences were confirmed by nucleotide sequencing using an Applied Biosystems 373A automated DNA sequencer and dideoxy terminator chemistry. The sequencing primers were designed using the Oligo 4.0 program (National Bioscience, Plymouth, MN). The nucleotide sequence of the α₉ and β₃ cDNAs were identical to the fibroblast α₉ (20) and HEL cell β₃ (21) sequences, respectively. The cDNAs were ³²P-radioabeled using the TAG-IT kit (Bios Corp., New Haven, CT) and used to screen the human osteoclastoma cDNA library in situ plaque hybridization under low stringency conditions as described (22).

Positive clones were plaque-purified, and cDNAs were excised to yield recombinant Bluescript plasmids in Escherichia coli XL1-Blue cells, according to methods recommended by Stratagene. Several partial positive clones were ligated together to generate full-length cDNAs; and three clones, pHOCα₉, pHOCβ₃ₐ, and pHOCβ₃c, encoding the α₉, β₃ₐ, and novel β₃c forms, respectively, were completely sequenced and used for further analysis.

**Genomic PCR—** To determine the splicing mechanism by which the variant β₃ (PHEOCβ₃c) is generated, PCR reactions were carried out using three sets of primers (see Fig. 4), DNA isolated from the β₃ genomic clone (P1 clone encompassing the entire β₃ coding region and flanking regions; kind gift of Dr. M. Ponzc, University of Pennsylvania, Philadelphia, PA) as a template, and eLONGase enzyme mix under conditions recommended by the manufacturer (Life Technologies, Inc.). The PCR products were gel-purified and subcloned into PCR2000 vector (Invitrogen, San Diego, CA), and the nucleotide sequence of the 5′- and 3′-ends were determined.

**Northern Analysis—** The analysis was performed using mRNA derived from human osteoclastoma tissue samples. Two-microgram aliquots of poly(A)⁺ RNA from an osteoclast-rich fraction and an osteoclast-depleted stromal cell fraction were fractionated on a 1% agarose formaldehyde gel and transferred to a nitrocellulose membrane (23). The Northern hybridization reaction was performed as described (24). Probes for the β₃ₐ and β₃c subunits (unique regions of the cytoplasmic domain and 3′-UTR regions; Fig. 3A) were generated by PCR and ³²P-radioabeled using the TAG-IT kit.

**RT-PCR Reactions—** Nested PCR reactions were carried out under standard conditions with β₃c-specific primer sets 1.3 and 2.4; see Fig. 3A) or β₃c-specific primers (5.7 and 6.8) and 5 ng of double-stranded cDNA prepared from heart, liver, lung, placenta, and prostate tissues or 1 µl of brain, osteoclastoma, osteoblast, stromal cells, or bone marrow cell cDNA libraries as templates (commercially prepared by Stratagene, La Jolla, CA in the Uni-ZAP XR vector). The PCR products were analyzed on a 1.2% agarose gel.

**Cells and Cell Culture—** Human embryonic kidney cells (HEK293 cells) were obtained from ATCC (catalog number CRL 1573). Cells were grown in Earl's minimal essential medium containing Earl's salts, 10% fetal bovine serum, 1% glutamine, and 1% penicillin-streptomycin.

**Constructions and Transfections—** A 3.2-kb EcoRI-KpnI fragment of the α₉ subunit, a 2.4-kb XhoI-XhoI fragment of the β₃ₐ subunit, and a 2.9-kb XhoI-XhoI fragment of the variant β₃c subunit containing the entire coding region were inserted into the EcoRI site of the pcDNA vector (Invitrogen, San Diego, CA) by blunt end ligation. The same constructs were also subcloned into the EcoRI-EcoRI cloning sites of the pC DNS vector (25), which contains a cytomegalovirus promoter and a G418-selectable marker. The pcDNA constructs were used for transient expression, and the pCDN constructs were used for stable expression. Transient transfections of the α₉, β₃ₐ, β₃c, α₉ and β₃c constructs into HEK 293 cells were carried out using the calcium phosphate precipitation method (26). For stable expression, 10⁶ 10⁵ cells/plate). After 48 h, the growth medium was supplemented with 450 µg/ml Geneticin (G418 sulfate; Life Technologies). The cells were maintained in selection medium until the colonies were large enough to be assayed.

**Analysis of β₉ and β₃c Transfectants by Flow Cytometry—** To determine whether the HEK 293 transfectants expressed the vitronectin receptor, the cells were reacted with 23C6 (28), a monoclonal antibody

HEK 293 cells were electrophoresed with α₉ + β₃ₐ or α₉ + β₃c, constructs (20 µg of DNA of each subunit) using a Gene Pulser (27) and plated in 100-mm plates (5 × 10⁶ cells/plate). After 48 h, the growth medium was supplemented with 450 µg/ml Geneticin (G418 sulfate; Life Technologies). The cells were maintained in selection medium until the colonies were large enough to be assayed.

**Analysis of β₉ and β₃c Transfectants by Flow Cytometry—** To determine whether the HEK 293 transfectants expressed the vitronectin receptor, the cells were reacted with 23C6 (28), a monoclonal antibody

**Fig. 1. Alignment of the cDNA and deduced amino acid sequences of the cytoplasmic regions of the β₃α₉ and β₃c subunits.** The positions indicate where the two sequences become divergent. The boxed region represents the putative transmembrane domain. Amino acids are indicated in single-letter code. The cDNA sequences of the 3′-UTRs have also been aligned for comparison.

**Fig. 2. Deduced amino acid sequences of several integral β₁ subunit cytoplasmic tails obtained from published data are compared with the β₃c cytoplasmic sequence.** Residues in boldface type represent the NPXY and NXXY motifs.
Fig. 3. A, schematic representation of the \( \beta_{3A} \) and \( \beta_{3C} \) cDNAs indicating regions of alternate splicing. The location and direction of priming of each of the oligonucleotide primers used for RT-PCR analysis are indicated by arrows. Oligonucleotides 1 (5'-GGCCAGGTCAATCACC-3'), 2 (5'-TCATTAGCCTGACTG-3'), 3 (5'-CTCTATCTAGCTGCCTTG-3'), and 8 (5'-GAAAGGGCACAAGCTGCTG-3') represent the nested set of primers that generates the \( \beta_{3C} \)-specific 530-bp fragment. Oligonucleotides 5 (5'-TATGCACACTGCTGAG-3'), 6 (5'-CTTAACCATTCATACCC-3'), 7 (5'-CTTATGTTTGACGGGAC-3'), and 8 (5'-GAAAGGGCACAAGCTGCTG-3') represent the nested set of primers that generates the \( \beta_{3A} \)-specific 470-bp fragment. B, Northern blot analysis of mRNA isolated from osteoclast-rich and stromal cell fractions of the osteoclastoma tissue. Two \( \mu \)g of the osteoclast-rich poly(A)\(^+\) RNA (lane 1) and two \( \mu \)g of stromal cell poly(A)\(^+\) RNA (lane 2) were electrophoresed on a 1% agarose-formaldehyde gel, blotted onto a nitrocellulose membrane, and probed with a \( ^{32}P \)-labeled \( \beta_{3C} \)-specific 470-bp fragment. C, detection of mRNA transcripts coding for the \( \beta_{3A} \) and \( \beta_{3C} \) subunits by the RT-PCR method. 530-bp \( \beta_{3C} \)-specific fragments are shown in the top part, and the 470-bp \( \beta_{3A} \)-specific fragments are shown in the bottom part. The various lanes represent different human tissues/cell lines used for nested PCR. Lane 1, brain; lane 2, heart; lane 3, lung; lane 4, liver; lane 5, kidney; lane 6, prostate; lane 7, placenta; lane 8, testis; lane 9, osteoclast-enriched fraction; lane 10, osteoblasts; lane 11, stromal cell fraction; lane 12, bone marrow cells; lane 13, no template control; lane 14, 1-kb ladder (molecular weight marker).

Preparation of Osteoclastoma Tissue Sections—A Hacker cryostat (Fairfield, NJ) was used to cut 8-μm sections of human osteoclastoma tissue. These were placed on four-well multitip slides (Henley Ltd, Loughton, Essex, UK), air-dried and fixed in acetone for 2 min. The sections were wrapped in foil and stored at -20 °C until required.

Immunocytochemistry Using \( \beta_{3A} \) or \( \beta_{3C} \)-Reactive Antibodies—Antibodies specific for either the \( \beta_{3A} \) or \( \beta_{3C} \) subunits were prepared by injecting rabbits with synthetic peptides corresponding to the unique carboxy-terminal sequences of each receptor. For the \( \beta_{3A} \) subunit, the peptide sequence was CHYAQSLRKWNQPVSIDG. Specific reactivity with the antibodies was demonstrated using a standard indirect immunofluorescence method (29), using phosphate-buffered saline, 2% fetal calf serum as the washing buffer and either a TRITC-labeled goat anti-rabbit, or fluorescein isothiocyanate-labeled goat anti-mouse immunoglobulin as the second antibody (Sigma, Poole, UK). To determine the specificity of the primary antibodies for their antigens, controls were set up in which the antibodies were preincubated for 2 h with either 70 μg of each of the \( \beta_{3A} \) and \( \beta_{3C} \) antigens, 70 μg of an unrelated peptide, or vehicle alone.

Ligand Binding Studies—Echistatin (2000 Ci/mmol) was purchased from Amersham Corp. HEK 293 cells were maintained as monolayer cultures in 75- or 150-cm² flasks in Earl's minimal essential medium supplemented with 10% fetal calf serum and 0.1 mg/ml G418. Prior to...
the binding assay, transfected 293 cells were rinsed with RPMI medium and trypsinized until the cells dislodged. Cells were washed once with RPMI medium and resuspended in RPMI medium supplemented with 0.1 mM MnCl₂ and 0.1% bovine serum albumin at a concentration of 1.3 × 10⁶ cells/ml.

¹²⁵I-Echistatin binding assays were performed in a 96-well filtration plate assembly (Millipore Corp., Bedford, MA) using 0.65-μm hydrophilic durapore membranes (catalog number MADV-N65). The 293 cell suspension (0.1 ml) was incubated at 37 °C for 1 h with ¹²⁵I-echistatin in the absence or presence of unlabeled αVβ₃ antagonists. After the incubation, cell-bound ¹²⁵I-echistatin was separated from free ¹²⁵I-echistatin by filtration using a Millipore filtration manifold, followed by washing with ice-cold RPMI medium. The cell-associated radioactivity remaining on the filters was determined by γ-counting (COBRA γ-counter; Packard Instrument Co.).

**Cell Adhesion Studies**—Corning 96-well enzyme-linked immunosorbent assay plates were precoated overnight at 4 °C with 0.1 ml of human osteopontin (0.5 mg/ml in RPMI medium). The plates were then washed once with RPMI medium and blocked with 3.5% bovine serum albumin in RPMI medium for 1 h at room temperature. Transfected 293 cells were resuspended in RPMI medium supplemented with 20 mM Hepes, pH 7.4, 0.1 mM MnCl₂, and 0.1% bovine serum albumin at a concentration of 1 × 10⁶ cells/ml. A 0.1-ml aliquot of cell suspension was added to each well and incubated for 1 h at 37 °C, in the presence or absence of various αVβ₃ antagonists. Following incubation, 0.025 ml of a 10% formaldehyde solution, pH 7.4, was added, and the cells were fixed at room temperature for 10 min. The plates were washed three times with 0.2 ml of RPMI medium, and the adherent cells were stained with 0.1 ml of 0.5% toluidine blue for 20 min at room temperature. Excess stain was removed by extensive washing with deionized water. The toluidine blue incorporated into cells was eluted by the addition of 0.1 ml of 50% ethanol containing 50 mM HCl. Cell adhesion was quantified at an optical density of 600 nm on a microtiter plate reader (Titertek Multiskan MC, Sterling, VA).

**RESULTS**

Cloning of the α₃ Subunit from the Human Osteoclastoma Library—A 3.2-kb fragment encompassing the coding region of the α₃ subunit was used to probe 2 × 10⁶ clones of the human
Comparison of the cytoplasmic tail sequences of the individual integrin β subunits reveals a striking degree of structural homology (Fig. 2). Most contain the conserved motifs NPYX and NXX(Y/F), which produce tight β turns in the structure. One of these motifs, NPYX, has been shown to have an influence on the recruitment of transfected integrins into focal contacts (30). As shown in Fig. 2, the β3C cytoplasmic domain lacks the NPYX motif, and, rather than NXX(Y/F), it contains the sequence NXXV. The cytoplasmic domain of the β3AB integrin lacks both motifs.

**Northern Analysis and RT-PCR**—Northern analysis with β3A- and β3C-specific probes revealed that a 6.6-kb β3C transcript was expressed in both the osteoclast-enriched and stromal cell fractions of the osteoclastoma tissue (Fig. 3B). RT-PCR reactions with various tissues and cell lines yielded a 530-bp β3A-specific band (Fig. 3C) and a 470-bp β3C-specific band (Fig. 3C) in many of the tissues tested including heart, liver, lung, kidney, osteoclastoma, placenta, and stromal and bone marrow cells. Interestingly, only the β3C subunit was expressed in the prostate and testis, while the osteoblast cells expressed only the β3A subunit (Fig. 3C).

**Mapping of the β3 Genomic Clone**—Mature β3A protein is encoded by 14 exons, which range in length from 0.09 to 3.7 kb and are contained within approximately 46 kb of genomic DNA (31). The variant β3AB transcript from placenta arises due to nonsplicing of the final intron (31). The sequence of the variant β3C cDNA (Fig. 1) predicts that it should be generated by alternate splicing to yield a novel exon 14, because it is identical to the β3A sequence up to exon 13. We designed several sets of sense oligonucleotide primers in the exon 14 region of the β3A gene using the published sequence (31). The antisense oligonucleotide primers were designed in the novel β3C region. The sequence of three oligonucleotide primers that worked well are shown in Fig. 4. Long range PCR using oligonucleotide primers 1 and 3 yielded a product that was about 9.5 kb in length, whereas PCR reactions carried out with oligonucleotide primers 2 and 3 yielded a product that was about 6 kb in length (data not shown). The nucleotide sequence of the 5′-ends of the 9.5- and 6-kb fragments revealed exact identity to the expected regions of the published β3A genomic sequence, whereas nucleotide sequence of the 3′-ends revealed 100% identity to novel β3C sequence. Based upon these data, we conclude that the novel exon 14 for the β3C transcript is located about 6 kb downstream of exon 14 that encodes the β3A transcript (Fig. 4). The β3A and β3C-transfected HEK 293 Cells—To allow characterization of this novel integrin subunit, HEK 293 cells were stably co-transfected with expression vectors for αv and β3C (HEKβ3C). For comparison, cells were also co-transfected with αv and β3A subunits (HEKβ3A). HEK 293 cells were selected because they do not endogenously express the β3 integrin. Instead, these cells express multiple β3 integrins, including αvβ1, αvβ3A, αvβ5, and αvβ6 (32). To monitor expression of the transfected receptors, flow cytometry was performed using murine monoclonal antibody, 23C6. No reactivity was detected in either population of cells that were reacted with either the IgG1 isotype or medium controls. Approximately 97% of the αvβ3A-transfected cells showed receptor expression, and this was stable (≥90% expression) for multiple (>20) passages. In contrast, flow cytometric analysis of HEKβ3C cells indicated that many cells within the population did not express receptor. To obtain a pure population of cells expressing the αvβ3C receptor, HEKβ3C cells were sorted by fluorescence-activated cell sorting, using 23C6 to label the receptor. After several passages, however, only approximately 60% of the previously sorted αvβ3C cells demonstrated positive reactivity with 23C6. This suggested that either the cells were rapidly losing expres-
demonstrated some background reactivity with the β3 subunit. Two stably transfected cell populations in culture were also quite different. HEK 293 cells were transiently transfected with vector, β3a, alone, β3a alone, or the αv in conjunction with either β3a or β3A. The adherent cells were stained with toluidine blue. Cell adhesion was quantified at an optical density of 600 nm.

### Inhibition of adhesion of the HEK293 cells cotransfected with the αv and β3A cDNAs by various inhibitors

Transfected cells were added to the rat osteopontin-coated wells in the presence or absence of various inhibitors, which included LM609 (60 μg/ml), 23C6 (100 μg/ml), and SK&F-107260 (1 μM). These data indicate that under the conditions used cell death occurred at a higher rate in the β3A-transfected cells compared with the β3A-transfected cells. Characteristics of the two stably transfected cell populations in culture were also quite different. HEKβ3A cells grew as a monolayer (see Fig. 6, C and D), while HEKβ3A grew as large aggregates of cells (Fig. 6, E and F).

### Immunocytochemistry—Antibodies were prepared against synthetic peptides corresponding to the sequences of the β3A and β3A cytoplasmic domains. When tested against the β3A- or β3A-transfected HEK 293 cells, both antibodies reacted strongly against their respective transfectants (Fig. 6, C and E), and this reactivity could be competed with the relevant peptide antigens (Fig. 6, D and F). The β3A antibody also demonstrated some background reactivity with the β3A transfectants that could not be competed out with either the β3A or the β3A peptides. In contrast, no reactivity could be detected on the wild type β3A transfectants that had been probed with the variant β3A antibody.

In osteoclastoma tissue, antibody to the β3A subunit demonstrated plasma membrane-associated reactivity on osteoclasts (Fig. 6A), similar to that of the 23C6 monoclonal antibody (data not shown). This reactivity was competed with the β3A peptide antigen (Fig. 6B) but was not affected by the β3A peptide. No reactivity was detected with the variant β3A antibody on the three osteoclastoma preparations tested (results not shown).

### Cell Adhesion to Human and Rat Osteopontin—Previous studies of the β3A cytoplasmic domain suggest that it modulates integrin ligand binding affinities and/or cell adhesion and that more than one location is required for mediating these functions (33). Since the carboxyl-terminal 17 amino acids of the β3A subunit differ from those of β3A and HEKβ3A cells displayed altered cell-cell and cell-matrix interactions in culture, it was of interest to determine whether cells expressing αvβ3A showed different adhesion properties than cells expressing αvβ3A genes.

To address this, transiently transfected HEK293 cells were tested in cell adhesion assays using human or rat osteopontin as substrate. HEK293 cells transfected with vector and those transfected with either β3A alone or β3A alone showed minimal adhesion to rat osteopontin (Fig. 7). Cells co-transfected with β3A and αv displayed significant adhesion to osteopontin. Adhesion of these αv- and β3A-co-transfected cells on osteopontin...
was mediated primarily by $\alpha_b\beta_{3A}$, since it could be blocked effectively by the addition of $\alpha_b\beta$-specific monoclonal antibodies (LM609 or 23C6). This adhesion was also RGD-mediated, since it was blocked by a cyclic RGD peptide, SK&F-107260 (Fig. 8). In contrast, cells co-transfected with $\alpha_C$ and $\beta_{3C}$ showed no adhesion to either rat or human osteopontin.

$\beta_{3C}$ and $\beta_{3A}$ Integrins Display Similar Ligand Specificity—To examine whether the lack of adhesion of the $\alpha_C + \beta_{3C}$-co-transfected cells was due to their inability to bind ligand, we compared the binding properties of the receptors in an intact cell binding assay using $^{125}$I-echistatin as radioligand. As demonstrated in Fig. 9, A and B, cells co-transfected with either $\alpha_C + \beta_{3C}$ or $\alpha_C + \beta_{3A}$ bound $^{125}$I-echistatin saturably and with high affinity. No $^{125}$I-echistatin binding was observed in cells transfected with either vector alone or with the $\beta_{3C}$ or $\beta_{3A}$ cDNAs in the absence of $\alpha_C$ co-transfection. Scatchard analysis of the binding data of the $\alpha_C + \beta_{3C}$-co-transfected cells gave a linear fit, with a $K_d$ of 4.76 nM and a $B_\text{max}$ of 1.22 pmol/10$^6$ cells, which is equal to approximately 0.74 $\times$ 10$^6$ sites/cell (Fig. 9A). The $\alpha_C + \beta_{3C}$-co-transfected cells bound $^{125}$I-echistatin with a $K_d$ of 5.1 nM and a $B_\text{max}$ of 0.44 pmol/10$^6$ cells, which is equal to approximately 0.27 $\times$ 10$^6$ sites/cell (Fig. 9B).

Since $^{125}$I-echistatin bound $\alpha_C\beta_{3C}$ and $\alpha_C\beta_{3A}$-co-transfected cells with similar affinity, the $^{125}$I-echistatin binding assay was used to examine competition of various RGD-containing peptides to the two cell types. These included unlabeled echistatin and five cyclic and four linear RGD peptides, all of which are potent $\alpha_b\beta_3$ antagonists (34). Table I shows that each of the compounds displayed similar binding affinities for $\alpha_C\beta_{3C}$ and $\alpha_C\beta_{3A}$-transfected cells, indicating that both the $\alpha_C\beta_{3C}$ and the $\alpha_C\beta_{3A}$ integrins were correctly presented and had similar ligand specificities.

## DISCUSSION

It is becoming increasingly clear that integrins play a key role in a versatile and complex array of cell-cell and cell-matrix interactions and signaling events in cells. This wide variety in function is achieved mainly due to the existence of multiple $\alpha$ and $\beta$ subunits as well as the many alternate spliced forms of integrins, particularly of the $\beta$ subunit (1, 13, 35, 36). With this diversity, individual cell types can vary their adhesive properties by selective expression of certain integrins.

Indirect evidence suggested that osteoclasts may express unique forms of the vitronectin receptor, so we undertook the cloning and characterization of the $\alpha_C$ and $\beta_3$ subunits from a human osteoclastoma cDNA library. We obtained the $\alpha_C$ integrin that was cloned was identical to that previously described (20). However, in addition to the previously described $\beta_{3A}$ subunit (21), we also found an alternatively spliced $\beta_3$ subunit that contains a unique cytoplasmic domain. This $\beta_{3C}$ cytoplasmic domain differs from the previously described placental $\beta_{3M}$ sequence as well (13). Numerous studies have demonstrated that the cytoplasmic domain of the $\beta$ subunit mediates not only interaction with the cytoskeleton but is also responsible for further downstream signaling events (see Refs. 5 and 37). The $\beta_{3A}$ (35) and $\beta_{3B}$ (13) subunits arise due to the retention of the last intron, whereas the $\beta_{3C}$ (36), $\beta_{3C}$ (47), and $\beta_{3C}$ forms (Fig. 4) arise due to splicing in of new exons. It is of interest that most of the alternatively spliced forms of the $\beta_1$ and $\beta_3$ integrins identified so far have had altered cytoplasmic domains.

Comparison of the short cytoplasmic domains of $\beta$ integrins has demonstrated conserved motifs. The NPXY sequence is a highly conserved motif in the cytoplasmic domain of $\beta$-integrins and has been shown to play a fundamental role in integrin-mediated function (30, 37). All of the alternatively spliced forms, including the $\beta_{3C}$ form, lack this motif. Expression of these variant forms in cells has resulted in altered functional properties. For example, cells expressing the variant $\alpha_{1\beta_{3C}}$ or $\alpha_{3\beta_{3C}}$ bound fibronectin but did not localize in focal adhesions (38). HEK 293 cells expressing $\alpha_{1\beta_{3C}}$ showed altered morphology in culture, and, despite showing nearly identical binding properties to the $\alpha_{1\beta_{3A}}$ integrin, these cells failed to adhere to either human or rat osteopontin.

The in vivo role of this alternatively spliced integrin subunit is not clear. The poor growth of $\beta_{3C}$-transfected HEK293 cells, rapid loss of $\beta_{3C}$ expressing cells in culture, and enhanced cell death observed by flow cytometry suggest a potential role for this novel splice variant in the regulation of cell survival. Loss of cell contact with the extracellular matrix has been recognized to be associated with cell death (39–41). Such cell death has been termed “anoikis” (homelessness) (40) and has also been described for osteoclasts (42). Of interest, a splice variant of the $\beta_1$ integrin, $\beta_{1C}$, was recently shown to be associated with inhibition of cell cycle progression in transiently transfected cells (43). These authors also experienced difficulty in obtaining cells stably transfected with the splice variant. Based on our experiments with transfected cells, it is tempting to speculate that a switch from $\beta_{3A}$ expression to transient expression of the $\beta_{3C}$ subunit in vivo may induce cell detachment from the extracellular matrix and subsequent cell death. This would be facilitated by the continuous recycling of integrins by synthesis, processing, and transport to the cell surface followed by internalization, as has been described for the $\beta_1$ and $\beta_3$ integrins (44). Furthermore, since expression of this integrin variant would be expected to be transient, it may be very
difficult to demonstrate expression of this integrin in situ, as has been our experience. In osteoclasts, detachment and cell death occur at the end of the resorption phase and have been shown to be regulated by inhibitors of osteoclast activity such as integrin antagonists and bisphosphonates (42, 45). However osteoclasts are rare cells, and in addition Parfitt et al. (46) have estimated that apoptosis occurs in only 0.3% of osteoclasts in bone. Thus, it is highly unlikely that this event would be observed in random tissue sections. Further studies will be required to determine the function of the β₃c integrin subunit and whether it may play a role in cell survival. Based upon analogy with other integrin cytoplasmic domains, it will also be of interest to identify the proteins with which this novel cytoplasmic domain interacts and its effect upon cell signaling.

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