A Glanzmann's mutation in β3 integrin specifically impairs osteoclast function

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Osteoclastic bone resorption requires cell-matrix contact, an event mediated by the αvβ3 integrin. The structural components of the integrin that mediate osteoclast function are, however, not in hand. To address this issue, we generated mice lacking the *β3 integrin* gene, which have dysfunctional osteoclasts. Here, we show the full rescue of $\beta3^{-/-}$ osteoclast function following expression of a full-length β3 integrin. In contrast, truncated β3, lacking a cytoplasmic domain (hβ3Δc), is completely ineffective in restoring function to $\beta3^{-/-}$ osteoclasts. To identify the components of the β3 cytoplasmic domain regulating osteoclast function, we generated six point mutants known, in other circumstances, to mediate β integrin signaling. Of the six, only the $S^{752}P$ substitution, which also characterizes a form of the human bleeding disorder Glanzmann's thrombasthenia, fails to rescue $\beta3^{-/-}$ osteoclasts or restore ligand-activated signaling in the form of c-src activation. Interestingly, the double mutation $Y^{747}F/Y^{759}F$, which disrupts platelet function, does not affect the osteoclast. Thus similarities and distinctions exist in the mechanisms by which the β3 integrin regulates platelets and osteoclasts.

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Introduction

The osteoclast is a polykaryon of monocyte/macrophage lineage (1, 2). It differs from other members of this family by its capacity to resorb bone, an event which necessitates contact between the osteoclast and bone matrix. Once this proximity is achieved, bone-derived signals induce the osteoclast to undergo dramatic polarization eventuating in formation, at its interface with matrix, of a unique ruffled membrane which is the cell's resorptive organelle. Thus, the means by which the osteoclast recognizes bone and transmits matrix-derived intracellular signals is critical to the cell's capacity to resorb the skeleton.

Integrins are heterodimeric transmembrane proteins consisting of α and β subunits, which not only mediate cell-cell and cell-matrix interaction, but also act as signaling receptors (3). The integrin αvβ3 is expressed by osteoclasts, and blocking studies establish that binding of this complex to bone is essential to the resorptive process (4, 5). Consistent with this posture, $\beta3^{-/-}$ mice become progressively osteosclerotic with age, a phenomenon due to dysfunctional osteoclasts, which fail to adequately polarize and develop abnormal ruffled membranes (6, 7). In culture, these cells do not adequately organize their cytoskeleton, and thus fail to spread normally. When placed on whale dentin, cultured $\beta3^{-/-}$ osteoclasts only superficially excavate the surface and fail to generate normal resorptive lacunae (7).

Despite the critical role played by αvβ3 in skeletal resorption, the molecular mechanisms by which the integrin regulates osteoclast function are incompletely understood. For example, the occupied integrin activates c-src (8, 9), a molecule central to the osteoclast's capacity to organize its cytoskeleton and resorb bone (10, 11), but the components of the integrin mediating this event are unknown.

Using a retroviral strategy, we demonstrate full rescue of $\beta3^{-/-}$ osteoclast function with a full-length *β3* cDNA. This observation permitted us to ask if the β3 cytoplasmic domain, known to transmit intracellular signals in many cell types, is essential for osteoclast function. We find that, in contrast to the complete rescue achieved by full-length β3, deletion of the integrin subunit's cytoplasmic domain renders it completely ineffective in $\beta3^{-/-}$ osteoclasts. To identify the components of the β3 cytoplasmic domain regulating osteoclast function, we generated a series of point mutants known, in other circumstances, to mediate β3 integrin signaling. Of the six mutants, only the $S^{752}P$ substitution, which also characterizes a form of the human bleeding disorder Glanzmann's thrombasthenia (12), fails to rescue the spreading and resorptive capacity of $\beta3^{-/-}$ osteoclasts or activate c-src upon ligation.

Methods

Retrovirus vector construction. We used the ΔU3 retroviral vector to express the human β3 integrin (13). Using human β3 cDNA plasmid as a template, we performed PCR with the following primer pair: 5′-ATCCTCTAGACTGCCATGCGAGCGCGGCCGCGGCCCCGGCCGCTC-3′ and 5′-CTAGAGATCTTTAAGTGCCCCGGTACGTGATATTGGTGAAGG-3′. The PCR product was digested with XbaI and BglII and subcloned into the shuttle vector pBluescriptI-ISK (Stratagene, La Jolla, California, USA). The pSK-hβ3 shuttle vector was digested with XbaI and BglII to generate a 2.5-kb insert, which was cloned into the XbaI and BamHI cloning sites of ΔU3 to give rise to ΔU3-hβ3. The vector expressing human β3 lacking the cytoplasmic tail, ΔU3-hβ3Δc, was constructed using the primer pair: 5′-ATCCTCTAGACTGCCATGCGAGCGCGGCCGCGGCCCCGGCCGCTC-3′ and 5′-CTAGAGATCTTTATTTCCATATGAGCAGGGCGGCAAGGCCAATGA-3′.

Mutagenesis. A 150-bp coding sequence from NdeI to BglII in pSK-hβ3 was used as a mutagenesis cassette. All mutations were generated using the QuickChange Site–directed Mutagenesis Kit (Stratagene). The mutated sites were confirmed by sequencing. The 150-bp fragment containing the desired mutation(s) was then released from the mutagenesis cassette by double digestion with NdeI and BglII, and then used to replace the 150-bp wild-type (WT) sequence of pSK-hβ3. The complete reconstituted full-length β3 cDNA with the desired mutation was subcloned into ΔU3 vector as described above.

Preparation of retrovirus. 293GPG packaging cells were cultured in DMEM with 10% heat-inactivated FBS supplemented with puromycin, G418, and tetracycline as described (13). ΔU3-hβ3 or its mutants were purified by CsCl gradient centrifugation. ΔU3-hβ3 and ΔU3-hβ3Δc were cotransfected with a plasmid encoding hygromycin into 293GPG cells using LipofectAmine Plus (Life Technologies Inc., Rockville, Maryland, USA). Hygromycin-resistant stably transfected clones were selected for 2 weeks in media containing 100 μg/ml Hygromycin B (Sigma Chemical Co., St. Louis, Missouri, USA). The clones producing highest titer of virus, as determined by percent transduction of bone marrow macrophages (BMMs), were expanded, and virus-bearing supernatant was harvested under antibiotic-deficient conditions. Virus from the stable transfectants was used for ΔU3-hβ3 and ΔU3-hβ3Δc. The vectors encoding point mutants were transiently transfected into 293GPG cells using LipofectAmine Plus. Virus was collected at 48-, 72-, and 96-hour time points after transfection.

Infection of the BMMs. Macrophages were isolated from bone marrow of 4- to 8-week-old $\beta3^{+/+}$ or $\beta3^{-/-}$ mice, cultured overnight in α-MEM containing 10% heat-inactivated FBS, and subjected to Ficoll-Hypaque (Ficoll; Sigma Chemical Co.; Hypaque 76; Nycomed, Princeton, New Jersey, USA) gradient purification as described (14). Cells at the gradient interface were collected and cultured in the presence of 10 ng/ml recombinant M-CSF (R&D Systems Inc., Minneapolis, Minnesota, USA) in suspension in Teflon beakers (Fisher Scientific, Pittsburgh, Pennsylvania, USA) for 2 days. Cells were then transduced with virus for 24 hours in the presence of 20 ng/ml recombinant murine M-CSF and 8 μg/ml polybrene (Sigma Chemical Co.), without antibiotic selection. Transduced cells were grown an additional 2–3 days in suspension prior to analysis of expression or osteoclastogenesis.

In vitro generation of osteoclasts. Uninfected or infected marrow macrophages were cultured in α-MEM containing 10% heat-inactivated FBS with ST2 cells in presence of 1×10^{-8} M 1,25-$(OH)_2$ vitamin D_3 and 1×10^{-6} M dexamethasone in 24-well tissue culture plates (1×10^5 BMMs and 1×10^4 ST2 cells per well). Under these conditions, osteoclasts begin to form at days 6–7. The cultures were stained for tartrate-resistant acid phosphatase (TRAP) activity at days 8–10. Percent area covered by spread osteoclasts was determined using OsteoMeasure software (Osteometrics, Decatur, Georgia, USA).

Bone resorption. Osteoclasts were generated on whale dentin slices from infected or uninfected marrow macrophages as described above. Dentin slices were harvested at days 8–10. Cells were removed from the dentin slices with 0.25 M ammonium hydroxide and mechanical agitation. Dentin slices were then subjected to scanning electron microscopy (15). Maximum resorption lacunae depth was measured using a confocal microscope (Microradiance; Bio-Rad Laboratories Inc., Hercules, California, USA) as described (7). For evaluation of pit number and resorbed area, dentin slices were stained with Coomassie brilliant blue and analyzed with light microscopy OsteoMeasure software (Osteometrics).

Immunostaining. In order to avoid background associated with ST2 stromal cells, osteoclasts were generated from transduced and nontransduced precursors on glass coverslips in the presence of 25 ng/ml M-CSF and 40 ng/ml RANKL. After 8–10 days, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% TritonX-100, rinsed in PBS, and immunostained with 1A2, an mAb against human β3 at 10 μg/ml in 0.1% BSA/PBS (a gift of S. Blystone, Department of Cell and Developmental Biology, State University of New York (SUNY) Upstate Medical University at Syracuse, Syracuse, New York, USA), followed by Cy3-conjugated goat anti-mouse Ab (Chemicon, Temecula, California, USA) and Alexa488-phalloidin (Molecular Probes Inc., Eugene, Oregon, USA).

Flow cytometric analysis. Transduced macrophages were blocked with goat IgG (Sigma Chemical Co.) (50 μg/10^6 cells) for 20 minutes on ice, washed twice with HBSS, and incubated with the anti-β3 mAb 1A2 (10 μg/ml) for 45 minutes on ice. Cells were then rinsed and treated with FITC-conjugated goat-anti-mouse serum (Sigma Chemical Co.) for 30 minutes on ice. As a negative control, we used β3-deficient BMMs infected with ΔU3-nlsβ-gal (which encodes β-galactosidase) and treated as described above. Additionally, negative controls for each cell population were performed by incubating cells with secondary antibody alone. Finally, cells were suspended in 0.5 ml of

HBSS and analyzed on a Becton-Dickinson FACScan (Becton-Dickinson Immunocytometry Systems, Mountain View, California, USA).

Src activation. Transduced BMMs were grown in α-MEM with 10% FBS, in the presence of M-CSF and RANKL for 3 days, then starved overnight in medium containing 1% FCS, without cytokines. On the fourth day, cells were lifted with 10 mM EDTA (37°C for 5 minutes) and pipetting, followed by two washes in α-MEM/0.5% BSA. Cells were either maintained in suspension or plated on vitronectin-coated (10 μg/ml, 4°C overnight) plates for 1 hour at 37°C. Suspension cultures or adherent cells were then lysed, as described (9). Cleared lysates (60 μg/condition) were subjected to immunoprecipitation with PY99-agarose beads (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) at 4°C overnight, followed by immunoblot for p^{416}-src (Cell Signaling Technology, Beverly, Massachusetts, USA). As a loading control, 10 μg cleared lysate was analyzed by immunoblot with a monoclonal anti-src antibody (16).

Results

In vitro, osteoclasts generated from *β3*$^{-/-}$ BMMs develop an abnormal cytoskeleton manifested by failure to spread on culture dishes. Furthermore, mutant osteoclasts maintained on whale dentin slices excavate shallow resorption lacunae (7). Thus, we asked if expressing human β3 integrin (hβ3) rescues the abnormal phenotype of *β3*$^{-/-}$ murine osteoclasts. We chose the human integrin because (a) high affinity mAb's specific for hβ3 are available, and (b) the cytoplasmic domains of human and murine β3 are identical, making it very likely that hβ3 would be effective in murine cells.

Because authentic osteoclast precursors, namely primary BMMs, are difficult to efficiently transfect, we used a retroviral approach (13). To this end, we cloned the full-length *hβ3* cDNA into the ΔU3 retroviral construct. To determine if integrin-transmitted intracellular signals are essential to osteoclastic bone resorption, we also constructed a retrovirus vector encoding hβ3 lacking its cytoplasmic tail (hβ3Δc) (Figure 1a).

β3-deficient BMMs were transduced with ΔU3-hβ3 or ΔU3-hβ3Δc, and surface expression of each was analyzed by flow cytometry with an antibody specific for the extracellular domain of hβ3. ΔU3-β-gal served as negative control. Retroviral transduction with either hβ3 construct yields equivalent surface expression of the WT and mutant integrin (Figure 1b). Following confirmation of hβ3 and hβ3Δc expression, transduced macrophages were cocultured with ST2 stromal cells in osteoclastogenic conditions. Nontransduced WT and *β3*$^{-/-}$ BMMs served as controls. Eight days later, the cultures were analyzed for osteoclast expression of the β3 external domain and stained for TRAP activity, a marker of osteoclast differentiation (Figure 2a).

WT cultures consist of very large (150–400 μm diameter), well-spread TRAP-expressing, multinucleated cells. ST2 stromal cells either overlie or are pushed aside by these osteoclasts. *β3*$^{-/-}$ cultures also contain numerous TRAP-expressing multinucleated cells, a manifestation of the fact that αvβ3 is not essential for osteoclastogenesis (7). These osteoclasts, however, spread poorly and therefore appear much smaller. Occasional larger osteoclasts are present but they also fail to spread well or push ST2 cells aside. Expression of full-length hβ3 completely restores the spreading capacity of the mutant osteoclasts. In contrast, expression of the truncated hβ3Δc yields a culture indistinguishable from that containing nontransduced *β3*$^{-/-}$ cells. Immunofluorescent staining of mature osteoclasts derived from hβ3- and hβ3Δc-transduced precursors confirms that retroviral-driven expression of these cDNAs persists and localizes with fibrillar actin as BMMs undergo osteoclast differentiation (Figure 2b).

We next turned to the functional implications of the morphological rescue of β3-deficient osteoclasts. To this end, we generated osteoclasts on whale dentin slices, and after 8 days assessed resorption lacunae formation by scanning electron microscopy. *β3*$^{+/+}$ osteoclasts form well-demarcated deep resorption pits, while those excavated by cells lacking the integrin are substantially fewer in number, shallow, and poorly defined (Figures 2c and 5b). Reflecting their recovered spreading capacity, the resorptive activity of *β3*$^{-/-}$ cells transduced with full-length hβ3 integrin is

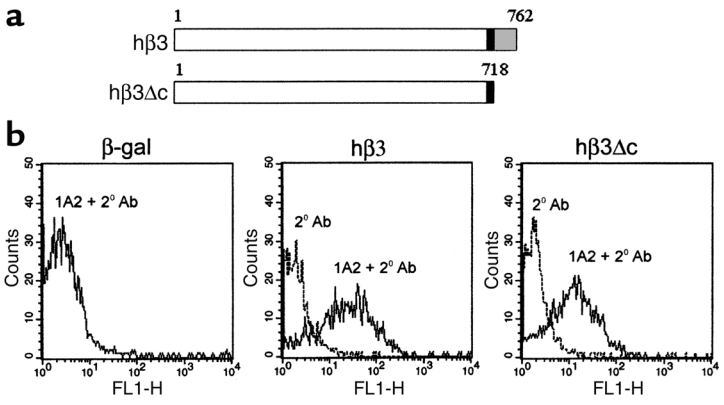

Figure 1

Efficient hβ3 integrin surface expression is obtained after retroviral transduction of primary osteoclast precursors. (**a**) Schematic of full-length and truncated hβ3 proteins produced by retroviral transduction of macrophages with ΔU3-hβ3 and ΔU3-hβ3Δc constructs. Black boxes, transmembrane domain; hatched box, cytoplasmic domain. (**b**) Flow cytometric analysis of *β3*$^{-/-}$ murine marrow macrophages transduced with virus bearing β-gal, hβ3, or hβ3Δc. The cells were subjected to FACS analysis using a mAb (1A2) recognizing human but not murine β3, and a FITC-conjugated secondary Ab (1A2 + 2°Ab). An internal negative control using secondary antibody alone (2°Ab) is shown in each panel. Cultures transduced with β-gal virus show no staining above background, while those transduced with either hβ3 or hβ3Δc show 80–90% of cells with significant integrin expression.

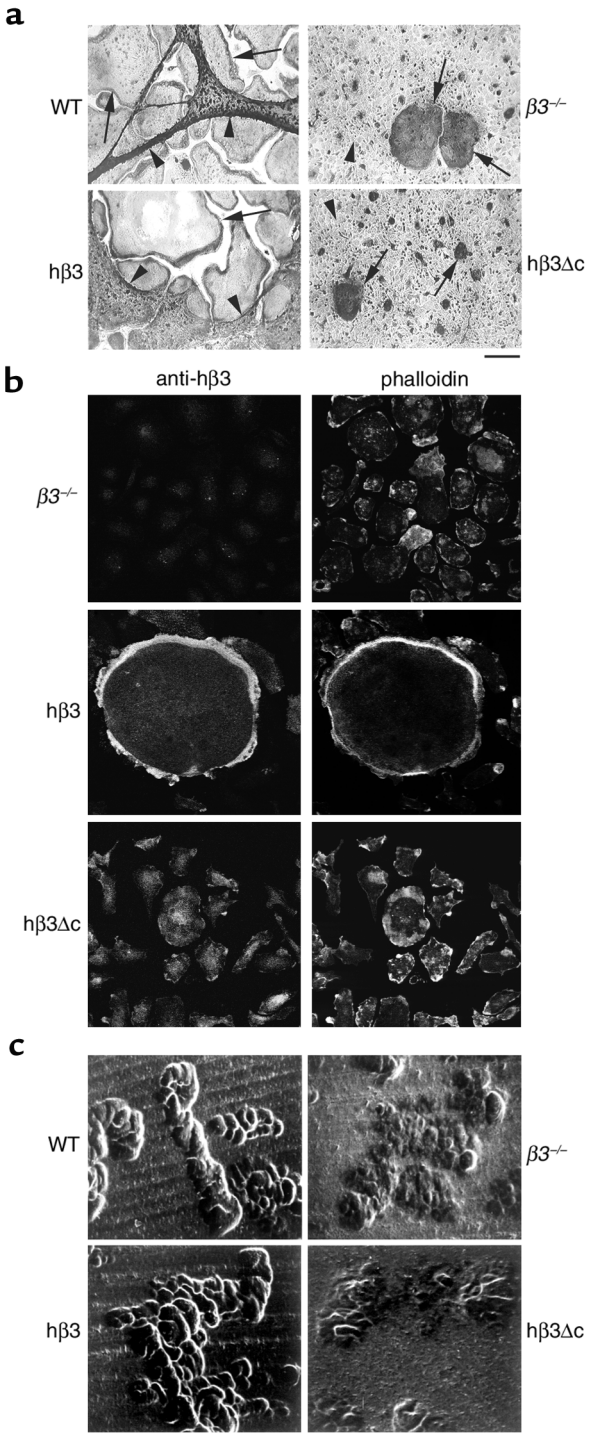

Figure 2
The β3 integrin cytoplasmic domain is essential for osteoclast spreading and resorptive activity. (**a**) TRAP-stained osteoclast cultures derived from WT and *β3*$^{-/-}$ marrow macrophages, and from *β3*$^{-/-}$ macrophages transduced with virus encoding hβ3 or hβ3Δc, in coculture with ST2 stromal cells (arrows, osteoclasts; arrowheads, ST2 stromal cells; scale bar = 100 μm). (**b**) *β3*$^{-/-}$ BMMs, either virgin or hβ3- or hβ3Δc-transduced, were cultured in osteoclastogenic conditions for 9 days with M-CSF and RANKL. Cultures were then immunostained with anti-hβ3 external domain mAb and incubated with Alexa488-phalloidin to visualize fibrillar actin. (**c**) Osteoclasts were generated as in **a**, on whale dentin. Eight days later, resorption lacunae were examined by scanning electron microscopy.

indistinguishable from their WT counterparts. In contrast, deletion of its intracellular tail abrogates the capacity of the integrin to restore the resorptive activity of β3-deficient cells.

Having established that the cytoplasmic domain of the β3 integrin is essential for osteoclast function, we turned to the individual amino acids mediating this event. On the basis of known regulatory capacity of αv-associated β integrin subunits in other systems (17–22), we mutated specific residues in the β3 cytoplasmic domain (Figure 3a). The mutants were cloned into ΔU3 retroviral construct, which was used to generate retrovirus for transduction of *β3*$^{-/-}$ osteoclast precursors.

Once again, osteoclasts generated from uninfected *β3*$^{-/-}$ BMMs fail to spread (Figure 3, b and c). In contrast, infection of *β3*$^{-/-}$ osteoclast precursors with hβ3, or any mutant, save one, restores the spreading capacity of their osteoclast progeny, establishing that these altered amino acids are not essential for organization of the osteoclast cytoskeleton. hβ3($S^{752}P$) is the only mutant which obviates rescue of *β3*$^{-/-}$ osteoclasts' capacity to spread. Flow cytometric analysis of transduced BMMs demonstrates that the failure of hβ3($S^{752}P$) to rescue spreading is not due to diminished surface expression of this mutant in osteoclast precursors (Figure 4a). Furthermore, immunofluorescent staining of mature osteoclasts demonstrates persistent expression of hβ3($S^{752}P$) and two other representative mutant β3 cDNAs (Figure 4b).

Mirroring spreading, the shallow, poorly-defined pits characteristic of *β3*$^{-/-}$ osteoclasts are completely normalized by hβ3 and representative, nondisruptive mutants, hβ3($D^{723}A$) and hβ3($Y^{747}F/Y^{759}F$) (Figure 5a). The lacunae formed by hβ3($S^{752}P$), in contrast, appear morphologically similar to those produced by *β3*$^{-/-}$ osteoclasts. Quantitative analysis reveals that the number of resorptive pits formed and the percent of dentin surface excavated by *β3*$^{-/-}$ osteoclasts transduced with hβ3 or hβ3($D^{723}A$) are indistinguishable from those generated by WT cells (Figure 5b). Alternatively, *β3*$^{-/-}$ cells bearing hβ3Δc, or the hβ3($S^{752}P$) mutant, mirror nontransduced *β3*$^{-/-}$ osteoclasts for these same indices of resorptive activity. Pit depth is also completely rescued in hβ3 and hβ3($D^{723}A$) cultures. Interestingly, while hβ3Δc and especially hβ3($S^{752}P$) transductants generate no more pits than do virgin *β3*$^{-/-}$ osteoclasts, they partially normalize pit depth ($P < 0.01$ compared with both WT and *β3*$^{-/-}$ osteoclasts).

We next turned to c-src activation, an intracellular signal mediated by αvβ3, in osteoclasts and asked if the event required the β3 cytoplasmic domain. Early osteoclasts were generated from *β3*$^{-/-}$ BMMs transduced with hβ3, hβ3Δc, or the hβ3($S^{752}P$) and hβ3($Y^{747}F/Y^{759}F$) mutants. The transductants were lifted with EDTA and kept in suspension or plated on the αvβ3 ligand vitronectin. After 1 hour, phosphorylation of c-src at the activation-specific Y^{416} site was determined by immunoblot (Figure 6). Adhesion to vitronectin activates c-src in osteoclasts generated from *β3*$^{-/-}$ marrow macrophages transduced with intact

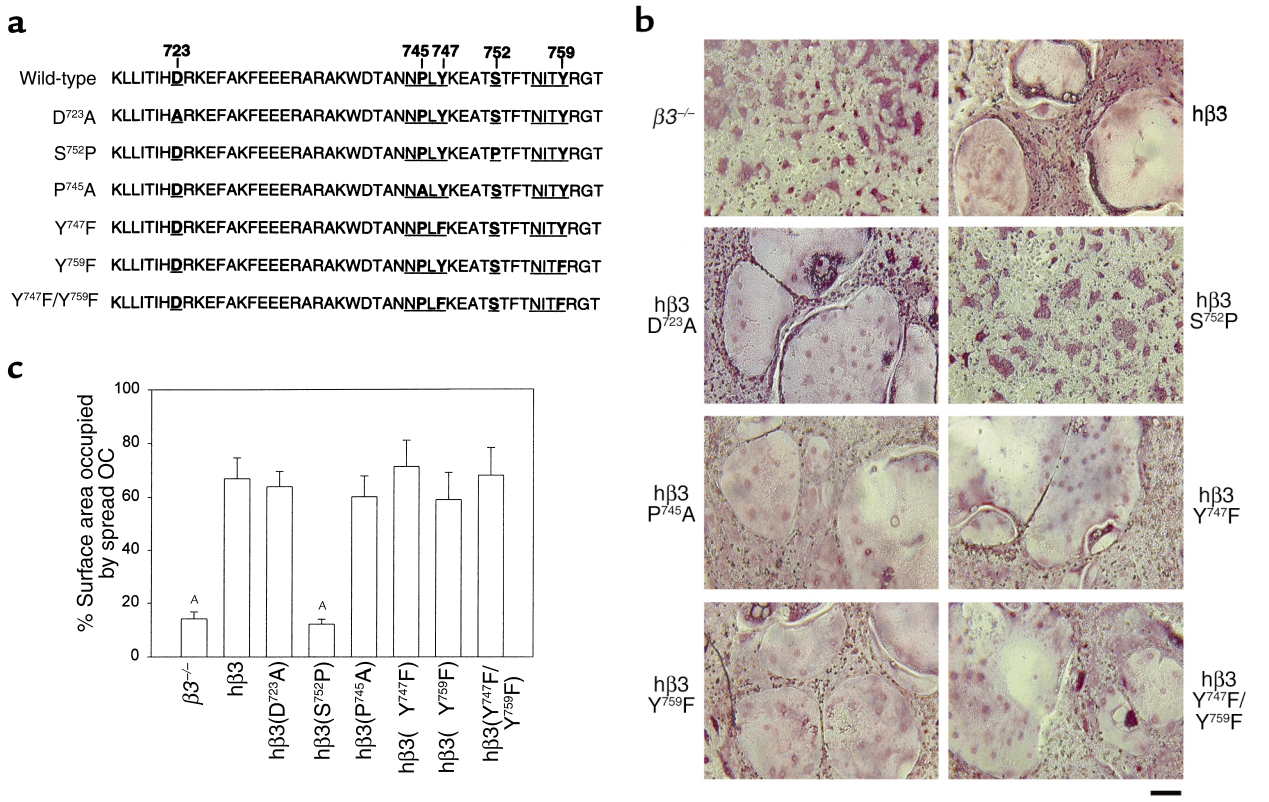

Figure 3
β3 integrin S^{752} uniquely regulates osteoclast spreading. (**a**) Sequences of six point mutants of β3 integrin cytoplasmic domain. (**b**) Osteoclasts derived from *β3*$^{-/-}$ macrophages infected with virus encoding WT hβ3 or mutations (detailed in **a**) were stained for TRAP activity after 8 days of ST2 coculture. Scale bar = 100 μm. (**c**) Percent surface area of culture covered by spread osteoclasts for the experiment shown in **b**. Results are typical of those seen in four separate experiments. $^{A}P < 0.001$ compared with hβ3 (without mutation); error bars represent SEM.

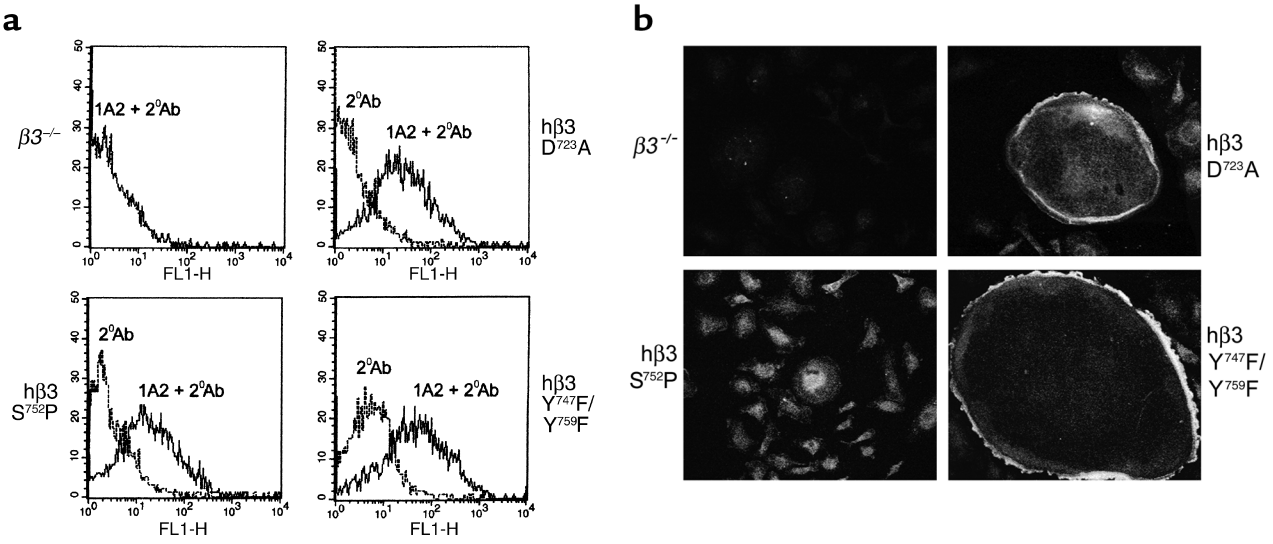

Figure 4
hβ3(S^{752}P) is effectively expressed by *β3*$^{-/-}$ osteoclasts and their precursors. (**a**) Flow cytometric analysis of *β3*$^{-/-}$ BMMs nontransduced or transduced with virus encoding hβ3(D^{723}A), hβ3(S^{752}P), or hβ3(Y^{747}F/Y^{759}F), for hβ3 expression using 1A2 (1A2 + 2°Ab). All mutants are expressed at approximately equivalent levels. An internal negative control using secondary antibody alone (2°Ab) is shown in each panel. (**b**) Mature osteoclasts, generated from the same transduced *β3*$^{-/-}$ precursors shown in **a**, were analyzed for expression of the various hβ3 mutants by immunofluorescence using anti-hβ3 external domain mAb.

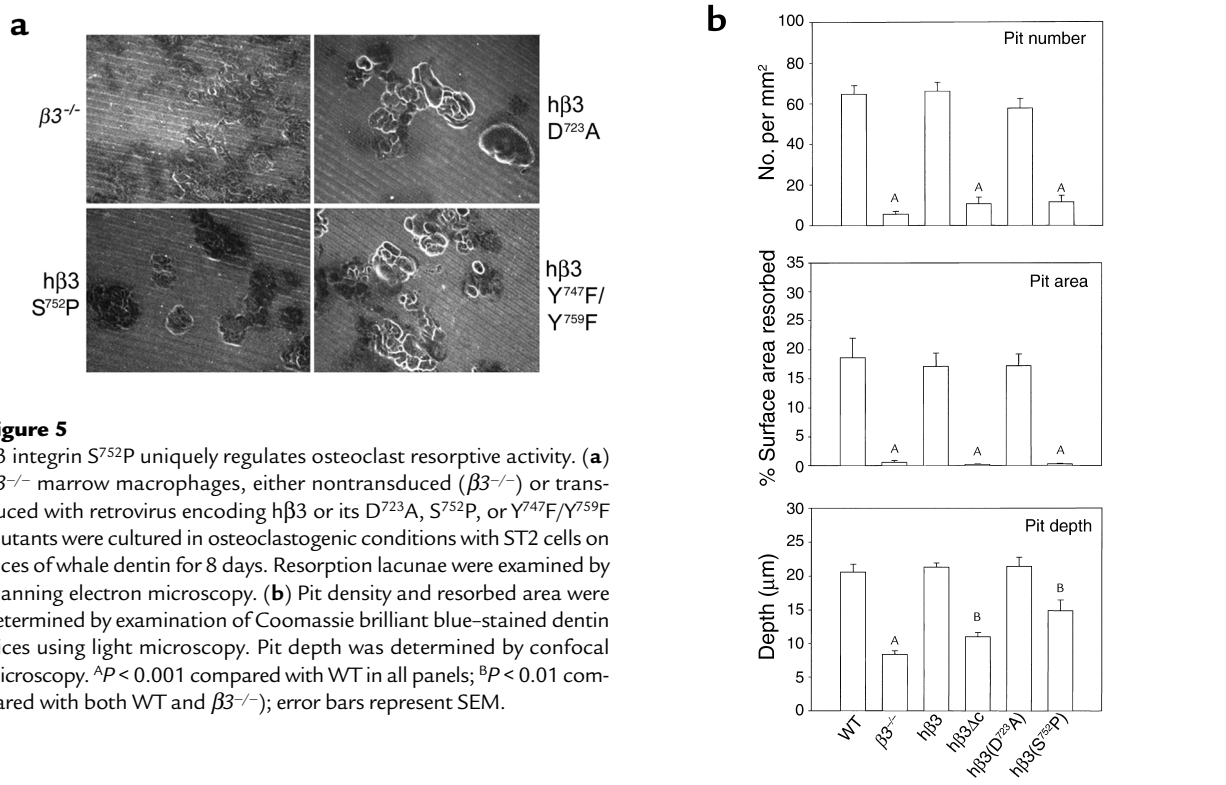

Figure 5
β3 integrin S^{752}P uniquely regulates osteoclast resorptive activity. (**a**) *β3*$^{-/-}$ marrow macrophages, either nontransduced (*β3*$^{-/-}$) or transduced with retrovirus encoding hβ3 or its D^{723}A, S^{752}P, or Y^{747}F/Y^{759}F mutants were cultured in osteoclastogenic conditions with ST2 cells on slices of whale dentin for 8 days. Resorption lacunae were examined by scanning electron microscopy. (**b**) Pit density and resorbed area were determined by examination of Coomassie brilliant blue–stained dentin slices using light microscopy. Pit depth was determined by confocal microscopy. $^{A}P < 0.001$ compared with WT in all panels; $^{B}P < 0.01$ compared with both WT and *β3*$^{-/-}$); error bars represent SEM.

hβ3 or hβ3(Y^{747}F/Y^{759}F). In contrast, no such activation occurs in osteoclasts bearing hβ3Δc and hβ3(S^{752}P), mirroring the functional effects of these mutations.

Discussion

Osteoclastic bone resorption is initiated by matrix recognition, and formation, at the cell-bone interface, of an isolated, acidified microenvironment, which is the site of skeletal degradation (1). This physical intimacy between the cell and bone indicates that attachment molecules on the osteoclast are pivotal to skeletal remodeling. This posture is buttressed by experiments performed, in vitro and in vivo, demonstrating that αvβ3 blockade blunts the osteoclast's ability to resorb bone (4–6). The clinical relevance of this observation is underscored by the capacity of soluble organic mimetics of the αvβ3 ligand to prevent experimental, postmenopausal osteoporosis (5).

With these experiments in mind, and the wish to determine the role of the αvβ3 integrin in skeletal development, we generated β3-deficient mice (7). Because the platelet integrin αIIbβ3 is not expressed in these animals, they serve as a model of the human bleeding dyscrasia Glanzmann's thrombasthenia (23). Reflecting osteoclast dysfunction, *β3*$^{-/-}$ mice are hypocalcemic and develop bone sclerosis as they age (7). While the bone phenotype in patients with Glanzmann's thrombasthenia is unknown, a reasonable possibility holds that they too may have increased bone mass. A likely clinical consequence of this phenomenon would be protection against pathological bone loss such as that attending cessation of ovarian function.

The fact that *β3*$^{-/-}$ osteoclasts fail to normally organize their cytoskeleton, in vitro and in vivo, represents compelling evidence that the integrin transmits matrix-derived signals essential to the resorptive process. Given that the majority of known signaling events mediated by αvβ3 depend upon the β3 cytoplasmic domain, we asked if such was the case regarding the osteoclast. To address this issue, we first expressed full-length hβ3 in *β3*$^{-/-}$ osteoclasts. This undertaking was complicated by the fact that primary macrophages, which are osteoclast precursors, cannot be transfected with high efficiency

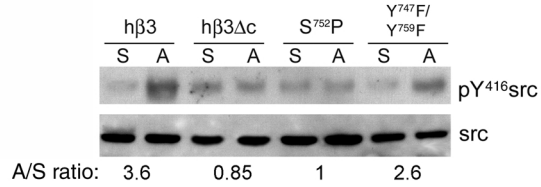

Figure 6
Activation of c-src requires the cytoplasmic tail of β3, and is abrogated by the S^{752}P mutation but not the Y^{747}F/Y^{759}F mutation. *β3*$^{-/-}$ BMMs transduced with hβ3, hβ3Δc, or the S^{752}P and Y^{747}F/Y^{759}F mutants were grown in M-CSF and RANKL for 3 days to generate early (not fully spread) osteoclasts. Following overnight starvation, cells were lifted with EDTA, and either kept in suspension (S) or adhered to vitronectin-coated plates (A) for 1 hour. As a control, cleared lysates were analyzed by immunoblot for total c-src. Activation of c-src was determined by immunoprecipitation of equal amounts of lysates with the anti-phosphotyrosine Ab PY99, followed by immunoblot with anti-pY^{416}src. The ratio of pY^{416}src band intensity between adherent and suspension cells (A/S) is indicated, normalized to total c-src levels.

by traditional methods. Thus, we utilized a retroviral strategy (13) which permits effective expression of the transgene in virtually all osteoclast precursors. These transduced cells, when placed in osteoclastogenic conditions, differentiate into osteoclasts indistinguishable from WT, both in their capacity to spread and, most importantly, to resorb bone. In contrast, $\beta3^{-/-}$ osteoclasts are unaltered by the β3 integrin transgene lacking the cytoplasmic domain. Thus, signal transduction mediated by the β3 cytoplasmic tail is critical for integrin function in the osteoclast.

Previous studies have demonstrated that binding of RGD-containing peptides to the integrin triggers various intracellular signals, including changes in intracellular calcium (24–26), and activation of c-src (8), PYK2 (9), p130cas (27), and PI3-kinase (28). Despite these observations, the structural components of the β3 cytoplasmic tail mediating osteoclast function have not been elucidated.

In an attempt to identify the amino acid residues in the β3 cytoplasmic tail critical to osteoclastic bone resorption, we generated single and double amino acid mutants based upon the demonstration, in other cells, that they alter β integrin function. D^{723} is implicated in forming a salt bridge with the α integrin subunit, thereby stabilizing an activated conformation (20). P^{745}, Y^{747}, and Y^{759} are located in two NPXY/NXXY motifs which are conserved among most β integrin subunits and mediate many aspects of integrin function (17–19). Mutation of integrin β1A-P^{781}, which corresponds to β3-P^{745}, dampens expression and inactivates β1A in the mouse embryonic stem cell line GD25 (22).

Of the six point mutants known to impact β integrin function, only $S^{752}P$ fails to rescue $\beta3^{-/-}$ osteoclasts. This mutation has also been documented in Glanzmann's thrombasthenia (29), in which platelets fail to aggregate. Thus, the residue regulating human platelet function also regulates the cytoskeletal organization and bone resorptive activity of osteoclasts. Specifically, hβ3($S^{752}P$) fails to rescue both the impaired capacity of $\beta3^{-/-}$ osteoclasts to spread, and the frequency with which they form resorptive lacunae. Similar to platelets (30), the effect of the mutation is specific for proline, as the more conservative mutation, $S^{752}A$, is as effective as WT hβ3 in rescuing osteoclast spreading (data not shown). This result suggests that local secondary structure, and not phosphorylation of S^{752}, likely mediates its central role in osteoclast function.

It is of interest that, like S^{752}, Y^{747} and Y^{759} are, in combination, also essential for platelet function (31). Both tyrosines, when phosphorylated, bind the signaling molecules SHC and GRB2, as well as the cytoskeletal protein myosin, in platelets (32, 33). Unlike S^{752}, however, these combined mutations fail to impact osteoclast function. Thus, the osteoclast and platelet may share some β3-mediated signaling pathways, while others appear cell-specific.

c-src is a tyrosine kinase essential for osteoclast function (10, 11, 34). The mechanism by which this proto-oncogene activates the osteoclast is complex, but clearly involves both the kinase and protein docking domains (35, 36). We find that αvβ3-dependent activation of c-src requires the cytoplasmic domain of β3, and is abrogated by the $S^{752}P$, but not the $Y^{747}F/Y^{759}F$, mutation. Thus, the ability of β3 to activate c-src correlates with its capacity to stimulate osteoclast function.

While the $S^{752}P$ mutation affects both osteoclast and platelet function, the downstream signaling mechanisms appear to be distinct. Deletion of c-src has not been reported to cause platelet dysfunction (34), in contrast to osteoclasts, and therefore is unlikely to represent the relevant β3-mediated signaling pathway in platelets.

Integrin signaling is bidirectional, with inside-out signals affecting ligand binding affinity, and outside-in signals determining intracellular events occurring upon integrin-ligand interaction (37). In the context of overexpression in Chinese hamster ovary (CHO) cells, Chen et al. (29) showed that the $S^{752}P$ mutation disrupts binding of the ligand-induced binding site (LIBS) antibody PAC1, suggesting a defect in inside-out signaling. In contrast, we find that σ osteoclasts expressing hβ3($S^{752}P$) bind AP5, another β3-specific LIBS antibody (R. Faccio, unpublished observations). Given that AP5 recognizes the activated form of αvβ3, this observation suggests that the β3($S^{752}P$) mutation, in osteoclasts, arrests outside-in signaling and thus, ligand-induced c-src activation.

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