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JCB Article

Dynamic changes in the osteoclast cytoskeleton in response to growth factors and cell attachment are controlled by β3 integrin

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The β3 integrin cytoplasmic domain, and specifically S^{752}, is critical for integrin localization and osteoclast (OC) function. Because growth factors such as macrophage colony–stimulating factor and hepatocyte growth factor affect integrin activation and function via inside-out signaling, a process requiring the β integrin cytoplasmic tail, we examined the effect of these growth factors on OC precursors. To this end, we retrovirally expressed various β3 integrins with cytoplasmic tail mutations in β3-deficient OC precursors. We find that S^{752} in the β3 cytoplasmic tail is required for growth factor–induced integrin activation, cytoskeletal reorganization, and membrane protrusion, thereby affecting OC adhesion, migration, and bone resorption. The small GTPases Rho and Rac mediate cytoskeletal reorganization, and activation of each is defective in OC precursors lacking a functional β3 subunit. Activation of the upstream mediators c-Src and c-Cbl is also dependent on β3. Interestingly, although the FAK-related kinase Pyk2 interacts with c-Src and c-Cbl, its activation is not disrupted in the absence of functional β3. Instead, its activation is dependent upon intracellular calcium, and on the β2 integrin. Thus, the β3 cytoplasmic domain is responsible for activation of specific intracellular signals leading to cytoskeletal reorganization critical for OC function.

Introduction

Osteoclastic bone resorption is a process requiring physical intimacy between the resorptive cell and bone matrix. Thus, cell–matrix attachment molecules, particularly integrins, play a central role in the capacity of osteoclasts (OCs) to degrade bone (Carron et al., 2000; Feng et al., 2001). The integrin αvβ3 is particularly important, in this regard, as its absence prompts OC dysfunction, eventuating in subnormal bone resorption and osteosclerosis (McHugh et al., 2000). The clinical significance of this observation is underscored by the fact that rats treated with an αvβ3 antagonist are spared the bone loss attending oophorectomy (Engleman et al., 1997).

While serving as a matrix attachment molecule in OCs, αvβ3 is also a signaling receptor (Eliceiri et al., 1998; Duong et al., 2000) that induces changes in intracellular calcium (Paniccia et al., 1993; Zimolo et al., 1994), protein tyrosine phosphorylation, and cytoskeletal reorganization (Clark et al., 1998; Schlaepfer and Hunter, 1998). The signaling capacity of integrins can be regulated by extracellular matrix molecules that, interacting with the integrin external domain, stimulate "outside-in" signaling (Takagi et al., 2002). Alternatively, "inside-out" signaling is induced by transactivated intracellular molecules, which interact with the cytoplasmic component of αvβ3 and prompt conformational changes in the integrin's ligand binding site. Outside-in and inside-out signals control the affinity state of the integrin, thereby modulating its binding capabilities and, ultimately, intracellular events (Pelletier et al., 1995; Geiger et al., 2001; Butler et al., 2003). Several cytokines and growth factors enhance integrin-dependent intracellular events, via inside-out signaling. Platelet-derived growth factor induces αvβ3-mediated adhesion in fibroblasts (Schneller et al., 1997), basic fibroblast growth factor augments migration in vascular endothelial cells (Kiosses et al., 2001), and macrophage colony–stimulating factor (M-CSF) and hepatocyte growth factor (HGF) modulate OC function, in part by increasing activated

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Key words: osteoclast; αvβ3 integrin; M-CSF; cytoskeleton; podosome

Abbreviations used in this paper: BMM, bone marrow macrophage; HGF, hepatocyte growth factor; LIBS, ligand-induced binding site; M-CSF, macrophage colony–stimulating factor; OC, osteoclast; OPN, osteopontin; VN, vitronectin.

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αvβ3 in the motile area of the membrane (Faccio et al., 2002).

In most cells, αvβ3 localizes with actin and other cytoskeletal proteins in focal adhesions (Ballestrem et al., 2001; Cukierman et al., 2001). OCs contain a related, but distinct, adhesive structure called the podosome, which consists of a core of F-actin bundles surrounded by a rosette-like structure containing αvβ3, vinculin, and α-actinin (Marchisio et al., 1988). As ligand activation of αvβ3 and growth factor stimulation promote podosome reorganization (Pfaff and Jurdic, 2001; Faccio et al., 2002), interest has turned to the intracellular components of the integrin and the signaling molecules linking it to cytoskeletal proteins.

c-Src is essential to OC function, as mice deleted of this tyrosine kinase develop osteopetrosis in the face of adequate numbers of dysfunctional OCs (Soriano et al., 1991). The fact that c-Src$^{-/-}$ OCs fail to organize a normal cytoskeleton suggests that c-Src may be a signaling molecule that associates with, and is activated by, αvβ3 (Duong et al., 2000; Sanjay et al., 2001). In addition, c-Cbl, a substrate of c-Src in OCs, is recruited to adhesion sites where it modulates the binding of the vitronectin (VN) receptor αvβ3 (Sanjay et al., 2001). Pyk-2, a member of the FAK family of kinases, is a signaling molecule that binds c-Src and c-Cbl, and appears to be essential for bone resorption. Pyk2 is activated when OCs are plated on ligands recognized by αvβ3 and is important for cytoskeletal organization during OC adhesion, migration, and sealing zone formation (Duong et al., 1998). The above compendium of events suggests that Pyk2 activation, in osteoclastic resorption, is mediated by αvβ3.

Rho family GTP-ases control cytoskeletal organization and dynamics and integrin-mediated signaling, as their inhibition blocks αvβ3-dependent motility (Clark et al., 1998; Ridley et al., 1999; Chellaiah et al., 2000; Ory et al., 2000). Importantly, Rho and Rac regulate the OC actin ring, and their blockade blunts the resorptive activity of the cell (Razzouk et al., 1999; Ory et al., 2000).

In the present study, we establish that the β3 cytoplasmic domain, specifically S^{752}, is responsible for organizing the OC cytoskeleton. Furthermore, like adhesion-dependent cytoskeletal reorganization, growth factor–induced αvβ3 inside-out signaling activates Rho GTPases. Finally, although αvβ3 is essential for activation of c-Src and c-Cbl, OCs lacking the integrin fully activate Pyk2.

Results

A functional β3 integrin cytoplasmic domain is required for its association with the actin cytoskeleton

Mice deleted of the β3 integrin subunit become osteosclerotic due to dysfunctional OCs, which fail to efficiently resorb bone (McHugh et al., 2000). As expected, the function of β3$^{-/-}$ OCs is rescued by transduction with retrovirus expressing full-length β3 cDNA. On the other hand, β3 lacking its cytoplasmic domain, or carrying a S^{752}P mutation, is incapable of rescuing OCs devoid of endogenous β3 (Feng et al., 2001). Furthermore β3$^{-/-}$ OC precursors cultured with RANKL and standard doses of

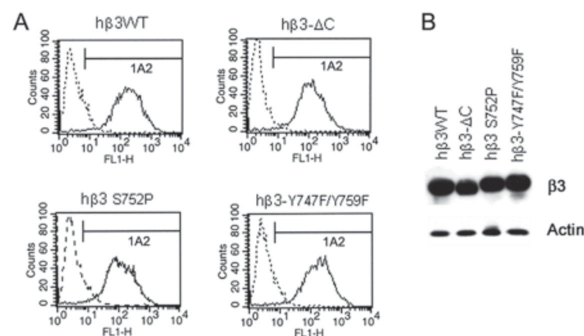

Figure 1. **Expression levels of β3 mutants by flow cytometry and Western blot.** (A) BMMs expressing hβ3 WT, hβ3-ΔC, hβ3 S^{752}P, or hβ3 Y^{747}F/Y^{759}F were incubated with a mAb against β3 integrin (1A2), followed by FITC-conjugated secondary antibody (solid lines). Cells with secondary antibody alone were used as negative control (dotted lines). (B) Mature OCs expressing the indicated β3 mutants were subjected to Western blot analysis using 7G2, a mAb against hβ3. Equal loading was confirmed by actin.

M-CSF fail to differentiate fully (Faccio et al., 2003). This osteoclastogenic defect can be rescued by increasing M-CSF concentrations, but the ability of these cells to resorb bone requires the presence of the integrin (Faccio et al., 2003). To further define the role that the β3 integrin cytoplasmic domain plays in organizing the OC cytoskeleton, we studied the distribution of podosomes in β3$^{-/-}$ OCs transduced with different human β3 integrin mutants. Four constructs were used for this purpose: the β3 integrin lacking its cytoplasmic tail (β3-ΔC); a mutant carrying the S^{752}P mutation or that bearing the OC nonsignificant double tyrosine mutation, Y^{747}F/Y^{759}F; and as positive control, the full-length human β3 (β3 WT). Equivalent levels of expression of all four constructs were confirmed by flow cytometry and by Western blot analysis (Fig. 1, A and B). Bone marrow macrophages (BMMs) expressing the indicated mutants were cultured on coverslips in the presence of RANKL (100 ng/ml) and a high dose of M-CSF (100 ng/ml), conditions that lead to the formation of completely spread OCs, even in nontransduced β3$^{-/-}$ cultures (Faccio et al., 2003). These cells cultured in high M-CSF are equivalent to their WT counterparts in terms of morphology, osteoclastogenic markers (Faccio et al., 2003), c-Fms (the receptor for M-CSF) levels, and pattern of integrin expression (see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200212082/DC1).

To delineate actin organization, OCs expressing the various mutants were immunostained with the anti–human β3 mAb 1A2 and costained with FITC–phalloidin (Fig. 2 A). The β3 integrin is organized in rosette-like structures, surrounding a core of F-actin bundles, in podosomes as previously described (Faccio et al., 2002). In nonresorbing OCs on glass, podosomes accumulate at high density at the periphery, yielding a row of actin dots flanked on each side by β3 integrin bands. This organization is present in β3$^{-/-}$ OCs bearing β3 WT or the β3 Y^{747}F/Y^{759}F mutation. Lack of functional β3, as in β3-ΔC and β3 S^{752}P mutants, completely abrogates the distribution of the integrin around the peripheral ring of F-actin, despite the normal appearance of

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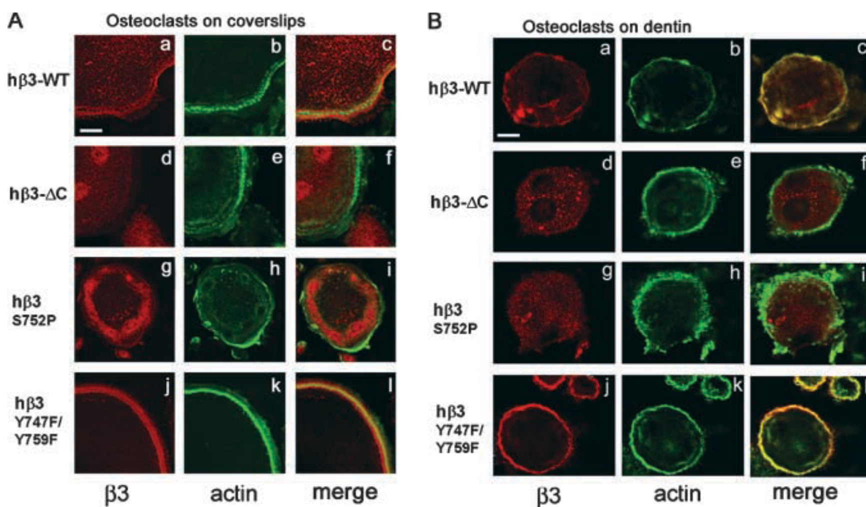

Figure 2. **S^{752} in the β3 cytoplasmic domain regulates integrin localization.** Mature $\beta3^{-/-}$ OCs transduced with the indicated β3 mutants were generated in the presence of RANKL and high dose M-CSF on glass coverslips (A) or dentin (B). Cells were stained with an anti–human β3 mAb (1A2) (red) and with FITC–phalloidin to detect actin distribution (green) and analyzed by confocal microscopy. Merged pseudocolored images obtained from the same confocal plane show the colocalization of β3 and F-actin in yellow. (A) OCs on coverslips bearing hβ3 WT or hβ3 $Y^{747}F/Y^{759}F$ organize αvβ3 (a and j) in a donut-like structure, around the F-actin core (b and k) of podosomes (merged on c and l). β3-ΔC (d and e) or the $S^{752}P$ mutation (g and h) fail to organize around the F-actin core, but are diffuse on the cell surface (merged on f and i). (B) β3 WT or β3 $Y^{747}F/Y^{759}F$ integrin (a and j) and actin (b and k) in OCs grown on dentin form well-defined, colocalizing rings (merged on c and l). In contrast, in β3-ΔC or β3 $S^{752}P$ mutants, the integrin (d and g) is diffusely distributed and fails to localize with the actin ring (e and h, merged in f and i). Bars, 5 μm.

the actin cytoskeleton. Similar results were obtained with another anti–human β3 mAb, AP3 (unpublished data). Interestingly other cytoskeletal components, including talin and vinculin, remain normally distributed in the podosomes of all mutants (see Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200212082/DC1).

To determine if the observations made on nonresorbing OCs are replicated in resorbing cells, the four species of β3-transduced OCs were generated on whale dentin and stained for the integrin and actin. Confocal microscopy reveals that the integrin, in dentin-resorbing β3 WT– and β3 $Y^{747}F/Y^{759}F$–expressing cells, localizes with the actin ring (Fig. 2 B). Once again, the β3-ΔC and β3 $S^{752}P$ mutants are diffusely distributed throughout the cell. This diffuse localization is not due to an increase in internalization, as the flow cytometric analysis shows similar surface expression levels to β3 WT and β3 $Y^{747}F/Y^{759}F$ mutants (Fig. 1 A).

External conformation of β3 integrin does not depend on the cytoplasmic domain

One possible explanation for the failure of β3-ΔC and β3 $S^{752}P$ to localize to podosomes is that the external domain of these mutants is not able to assume the activated, high-affinity conformation necessary for appropriate substrate interaction. One tool to assess the ability of β3 integrin to assume the activated conformation of the external domain is the anti–ligand-induced binding site (LIBS) antibody AP5. In low calcium buffer, this Ab binds all αvβ3 integrin on the cell surface, converting it to the active conformation. In high calcium buffer, AP5 binds only the integrin already in the activated state (Faccio et al., 2002). An increase in fluorescence intensity of αvβ3-expressing cells when AP5 binding is assessed in low calcium, relative to high calcium buffer, indicates that the external domain of the integrin can assume the activated conformation in response to the Ab. When pre-OCs transduced with β3-ΔC and β3 $S^{752}P$, as well as with β3 WT and β3 $Y^{747}F/Y^{759}F$, are analyzed in this manner, there is an increase in AP5 binding in low calcium, indicating that each of these β3 constructs undergoes conformational change (Fig. 3 A). Thus, the failure of the β3-ΔC and β3 $S^{752}P$ mutants to properly localize in podosomes does not reflect an inability of these integrins to assume an activated conformation.

Growth factor–mediated inside-out activation of β3 is dependent on its cytoplasmic domain

A second possible explanation for the aberrant localization of β3-ΔC and β3 $S^{752}P$ pre-OCs is that these mutants are not activated by signals emanating from the cell interior. One mechanism for such inside-out activation is growth factor treatment. We have previously shown that both HGF and M-CSF activate αvβ3 in this manner (Faccio et al., 2002). To determine whether the β3-ΔC and β3 $S^{752}P$ mutants are defective in growth factor–mediated activation, pre-OCs transduced with the β3 mutants were incubated with either HGF or M-CSF for 30 min before evaluation of the activated state of the integrin via AP5 staining in high calcium buffer. HGF and M-CSF activate β3 WT and β3 $Y^{747}F/Y^{759}F$, but fail to impact the integrin lacking the entire cytoplasmic domain (β3-ΔC) or bearing $S^{752}P$ (Fig. 3 B). Thus, the β3 cytoplasmic domain, and specifically S^{752}, is required for growth factor–induced formation of a high-affinity αvβ3 complex.

Localization of activated αvβ3 integrin to lamellipodia requires the β3 cytoplasmic domain

Upon growth factor activation, OC αvβ3 moves to the motile region of the cell membrane (Faccio et al., 2002). Having found that dysfunctional β3 mutants fail to properly localize in resting OCs, and do not become activated in response to growth factors, we determined if these mutants also fail to localize properly in the newly formed lamellipodia in response to growth factor stimulation. Thus, $\beta3^{-/-}$ OCs bearing WT β3, β3-ΔC, β3 $Y^{747}F/Y^{759}F$, or β3 $S^{752}P$ constructs grown on coverslips were exposed to HGF or M-CSF for 30 min and, after fixation, stained with AP5 (in high calcium) to detect localization of the activated form of αvβ3. Unstimulated OCs carrying β3 WT or the $Y^{747}F/Y^{759}F$ mutation express

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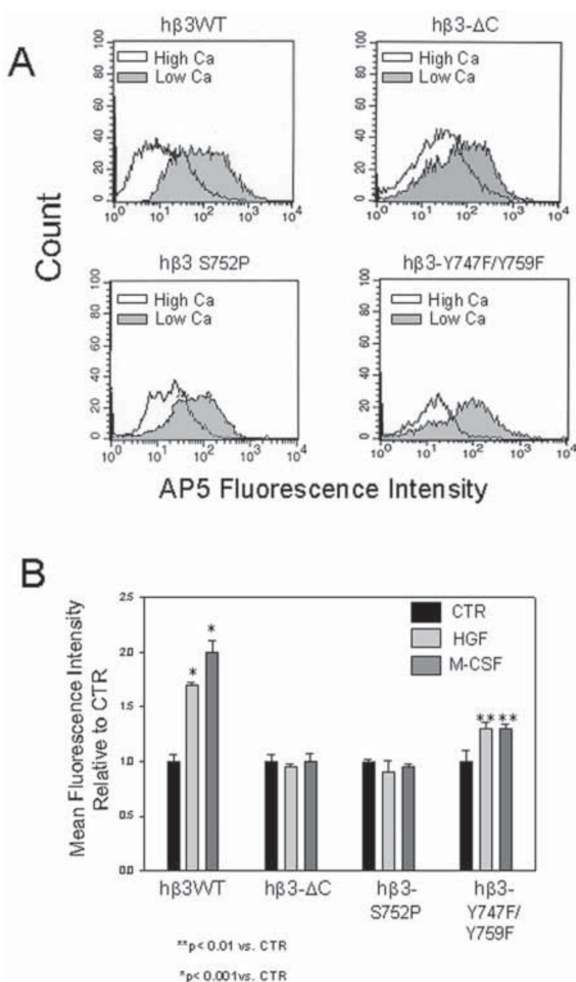

Figure 3. **β3 S^{752} regulates inside-out, but not outside-in, activation of αvβ3.** (A) $β3^{-/-}$ pre-OCs expressing the indicated mutants were incubated with AP5 (50 μg/ml) in low or high calcium and subjected to FACS® analysis. Results are presented as overlapping histograms for high (white) and low (gray) calcium treatments for each mutant. The tracings for high calcium buffer indicate basal activation of the integrin, as in this circumstance, AP5 binds previously activated αvβ3 integrin. A shift toward higher fluorescence intensity in low calcium buffer indicates that AP5 mediates integrin activation. In each group of transduced pre-OCs, incubation with AP5 in low calcium buffer yields a significant increase in mean fluorescence intensity, relative to high calcium buffer, indicating that activation by this anti-LIBS Ab from the cell's exterior does not depend on the β3 cytoplasmic domain. (B) Pre-OCs were preincubated with HGF (50 ng/ml, light gray bars) or M-CSF (100 ng/ml, dark gray bars) before staining with AP5 in high calcium buffer to determine the degree of growth factor–mediated integrin activation. Results are expressed as fold increase in the mean fluorescence intensity of AP5 staining compared with unstimulated control cells (CTR), arbitrarily designated as 1 for each untreated mutant.

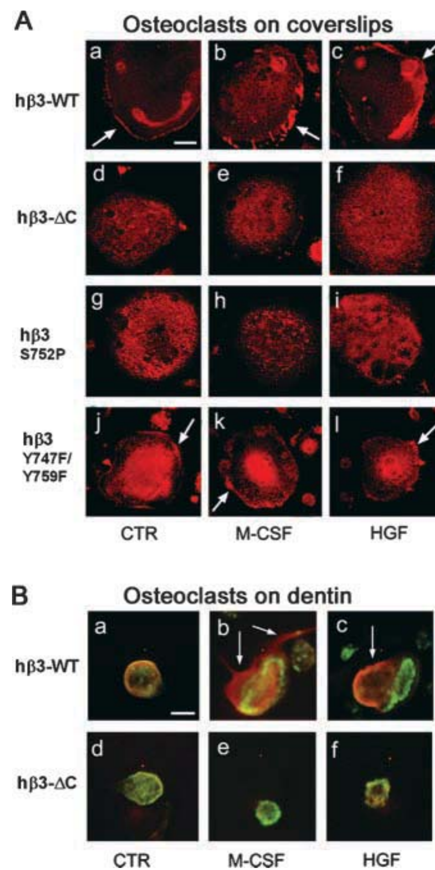

Figure 4. **Localization of αvβ3 in lamellipodia and membrane extensions is regulated by functional β3 cytoplasmic domain.** (A) Mature $β3^{-/-}$ OCs expressing the indicated β3 integrin constructs were either untreated (CTR) or stimulated with M-CSF or HGF to induce integrin activation, and stained with mAb AP5 (red), which detects only activated integrin. In control circumstances, cells expressing β3 WT (a) and β3 $Y^{747}F/Y^{759}F$ (j) present the activated integrin in lamellipodia (arrows). In contrast, in OCs expressing β3-ΔC (d) and β3 $S^{752}P$ (g), the activated integrin is randomly distributed throughout the cytoplasm. M-CSF or HGF clusters activated β3 WT (b and c, arrows) and β3 $Y^{747}F/Y^{759}F$ (k and l arrows) at the cell's edge, while β3-ΔC (e and f) and β3$S^{752}P$ (h and i) remain diffusely distributed. (B) $β3^{-/-}$ OCs expressing hβ3 WT or hβ3-ΔC cultured on dentin were stained with AP5 (red) and FITC–phalloidin (green). Shown are pseudocolored overlay images taken in the same confocal plane. β3 WT OCs stimulated with HGF or M-CSF form AP5-containing membrane extensions (arrows). In contrast, OCs expressing β3-ΔC show no response to the cytokines, despite their ability to form an actin ring. Bars, 10 μm.

the activated integrin along membrane ruffles and in lamellipodia (Fig. 4 A, CTR). Treatment with either growth factor induces AP5-positive membrane extensions (lamellipodia). This phenomenon is completely abrogated in β3-ΔC– and β3 $S^{752}P$–bearing cells, which are unable to spread and form lamellipodia in response to growth factors (Fig. 4 A). These observations are confirmed by counting the percentage of cells with multiple lamellipodia extensions (Table I). These data show that M-CSF and HGF induce lamellipodia in cells expressing β3 WT and β3 $Y^{747}F/Y^{759}F$ but not in those transduced with β3-ΔC or β3 $S^{752}P$.

We next turned to the effect of HGF or M-CSF on dentin-residing OCs, which represent resorptive cells. OCs expressing β3 WT or β3-ΔC were exposed to the individual cytokines and stained with AP5 (Fig. 4 B). In β3 WT–bearing cells, this exercise revealed that both cytokines induce membrane protrusions (arrows) that contain a predominance of activated αvβ3. Despite their ability to attach to bone and form actin rings on dentin, β3-ΔC OCs are unaffected by the growth factors, failing to spread and lacking membrane protrusions. Note that the activated integrin is

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Table I. **Lamellipodia formation in response to M-CSF and HGF is regulated by functional β3 cytoplasmic domain**

β3 constructs	CTR	+HGF	+M-CSF
WT	34 ± 5	73 ± 4[a]	70 ± 6[a]
ΔC	13 ± 7	25 ± 10	20 ± 10
$S^{752}P$	10 ± 5	14 ± 5	15 ± 6
$Y^{747}F/Y^{759}F$	20 ± 4	74 ± 8[a]	60 ± 5[a]

OCs bearing the indicated β3 constructs were cultured on coverslips, treated with M-CSF or HGF for 30 min, fixed, and stained with FITC–phalloidin to detect the actin organization. Ten 200× fields per coverslip were analyzed in triplicate. Data represent the percentage of cells with multiple lamellipodia extensions. M-CSF and HGF induce lamellipodia in cells expressing β3 WT and β3 Y747F/Y759F but not in those transduced with ΔCβ3 or β3 S752P.
[a]$P < 0.001$ vs. CTR.

only weakly localized in the actin ring, in all mutants, as previously described (Faccio et al., 2002, 2003).

Growth factors promote cytoskeletal rearrangement in a β3 integrin–dependent manner

The observation detailed in Fig. 4 led us to hypothesize that the β3 integrin controls cytoskeletal changes induced by growth factors. To address this issue, we stained unstimulated and growth factor–treated $\beta3^{+/+}$ and $\beta3^{-/-}$ OCs with FITC–phalloidin and analyzed actin organization by confocal microscopy. Once again, the absence of β3 integrin does not alter the peripheral podosomal distribution of F-actin in untreated OCs (Fig. 5 A, CTR). However, in the presence of HGF or M-CSF, F-actin moves from the podosomes to short filamentous protrusions, consistent with lamellipodia formation, only in $\beta3^{+/+}$ OCs (Fig. 5 A, low and high magnification; Table I). To confirm that this observation is an integrin-dependent consequence of podosome reorganization, we examined the distribution of α-actinin, a cytoskeletal protein involved in the formation and stability of podosomes, and a link between actin and integrins (Pavalko et al., 1991). α-Actinin distribution in $\beta3^{+/+}$ and $\beta3^{-/-}$ OCs mirrors that of actin, both in the presence and absence of growth factors (Fig. 5 A). Consistent with this finding, immunoblot analysis shows an increase in the pool of α-actinin present in the Triton X-100 soluble fraction exclusively in $\beta3^{+/+}$ OCs treated with HGF and M-CSF (Fig. 5 B). In $\beta3^{-/-}$ cells, α-actinin remains in the insoluble fraction. Similar results were obtained using OCs transduced with the different β3 mutants. Dramatic changes in the peripheral ring of actin are seen in response to M-CSF (Fig. 5 C), and the content of α-actinin in the Triton X-100 soluble fraction increases in β3 WT and

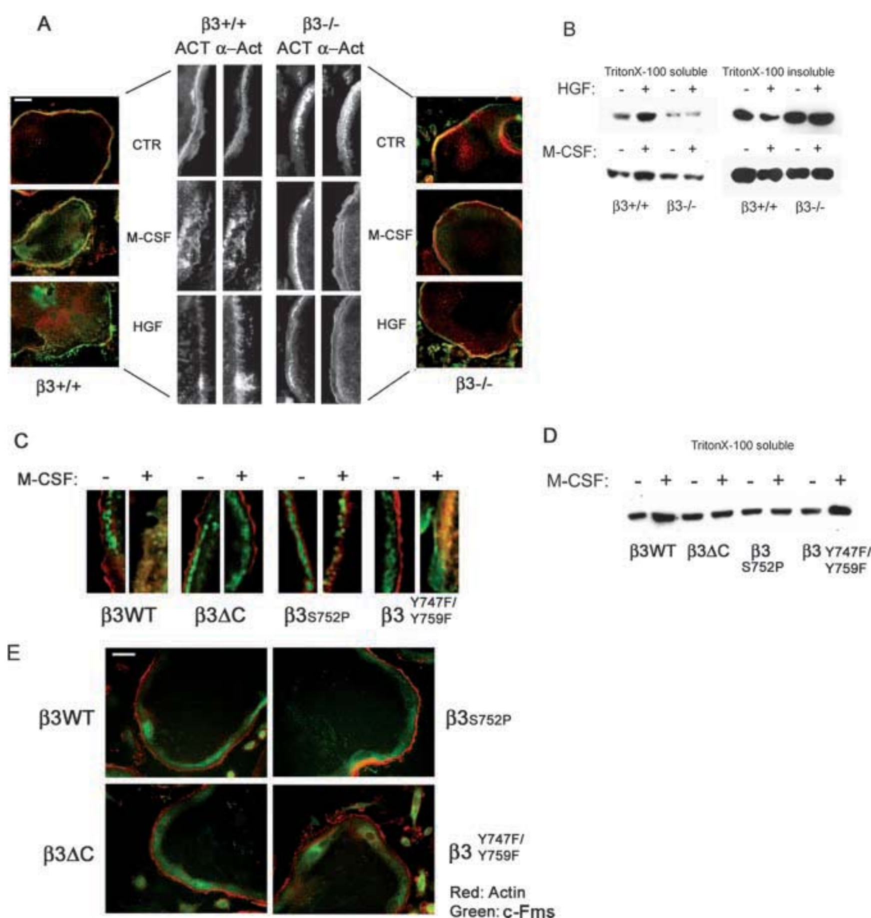

Figure 5. **HGF- and M-CSF–induced cytoskeletal reorganization is αvβ3 dependent.** (A) $\beta3^{+/+}$ or $\beta3^{-/-}$ OCs, cultured on coverslips, were treated with 100 ng/ml M-CSF or 50 ng/ml HGF for 10 min. Cells were stained with FITC–phalloidin for actin (green) and anti–α-actinin mAb (red). Although the absence of αvβ3 does not alter the peripheral ring of actin in untreated cells (CTR), redistribution of podosomes, along with α-actinin, into membrane extensions in response to growth factors is observed only in $\beta3^{+/+}$ OCs (pseudocolor overlays). High magnification images of the peripheral membrane (center) show the reorganization of actin (ACT) and α-actinin (α-Act) from podosomes to membrane ruffles in $\beta3^{+/+}$ but not in $\beta3^{-/-}$ OCs. Bar, 10 μm. (B) $\beta3^{+/+}$ or $\beta3^{-/-}$ OCs, treated as in A, were lysed in a Triton X-100 buffer, and soluble and insoluble fractions were separated by centrifugation. Immunoblot analysis was used to detect the distribution of α-actinin in the two fractions. (C) $\beta3^{-/-}$ OCs transduced with the indicated mutants were treated with M-CSF (A) stained for actin (green) or α-actinin (red). Overlay high magnification images show a redistribution of podosomes into newly formed membrane extensions only in β3 WT and β3 $Y^{747}F/Y^{759}F$ mutants. (D) Transduced OCs, treated as in C were lysed in a Triton X-100 buffer, and α-actinin distribution in the soluble fraction was analyzed by Western blot. (E) Immunolocalization of c-Fms in mature OCs expressing the indicated β3 mutants. Cells were stained for c-Fms (green) and costained with TRITC–phalloidin for actin (red). Overlays show similar distribution of c-Fms in all β3 mutants. Bar, 10 μm.

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β3 $Y^{747}F/Y^{759}F$ mutants, but not in those transduced with β3-ΔC or β3 $S^{752}P$ (Fig. 5 D). The failure of β3-null cells to respond to M-CSF is not dependent on different expression levels of c-Fms (Fig. S1) or on its different localization among the various β3 mutants (Fig. 5 E). These observations suggest that, in OCs, growth factor–induced reorganization of podosomes, leading to the formation of new membrane ruffles, is a β3-dependent event.

β3 integrin cytoplasmic domain governs OC adhesion and migration

The fact that β3-ΔC– and β3 $S^{752}P$–expressing OCs fail to spread in response to growth factors suggests that interaction of these mutant integrins with the extracellular matrix may be defective. To address this issue, we plated $β3^{-/-}$ pre-OCs bearing the various constructs on osteopontin (OPN), a substrate recognized by the αvβ3 integrin, or, as negative control, on BSA. Pre-OC adhesion to OPN is decreased threefold in cells carrying the β3-ΔC or $S^{752}P$ mutations, compared with β3 WT pre-OCs (Fig. 6 A), mirroring the defective adhesion of $β3^{-/-}$ cells (unpublished data). Furthermore, pretreatment with AP5, HGF, or M-CSF, all of which activate the integrin (Fig. 3), enhances the number of adherent β3 WT by 40–50%, and to a lesser extent β3 $Y^{747}F/Y^{759}F$–expressing cells (Fig. 6 A). On the other hand, these integrin-activating agents fail to impact the adhesion of β3-ΔC– and β3 $S^{752}P$–bearing pre-OCs. Similar results were obtained using VN as a substrate (unpublished data). In contrast, adhesion to native collagen, a β3 integrin–independent substrate, was similar in all mutants and was not enhanced by growth factors (unpublished data).

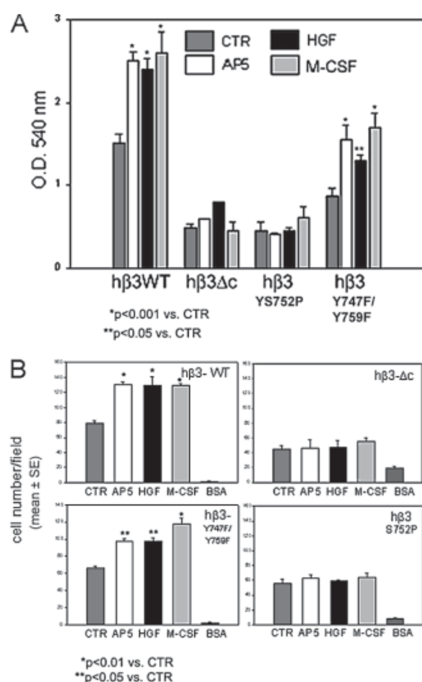

Figure 6. **Pre-OC adhesion and migration require functional β3 cytoplasmic domain.** (A) Adhesion assays to OPN were performed using $β3^{-/-}$ pre-OCs bearing the indicated β3 constructs. Adhesion to OPN is decreased threefold in cells carrying β3-ΔC or β3 $S^{752}P$ (CTR bars, dark gray) and is not affected by AP5 (light gray), HGF (black), or M-CSF (gray), which induce integrin activation. In contrast, the same factors increased adhesion of β3 WT– and β3 $Y^{747}F/Y^{759}F$–expressing cells by 40–50%. (B) Migration assay toward OPN was performed on the same cells as in A. Migration of untreated cells toward OPN (CTR, dark gray) is impaired in β3-ΔC and β3 $S^{752}P$ mutants. Moreover, AP5 (light gray), HGF (black), and M-CSF (gray) fail to rescue the directed mobility of these cells, whereas the same factors enhance the migratory capacity of β3 WT– and β3 $Y^{747}F/Y^{759}F$–expressing cells. *, $P < 0.01$ vs. CTR; **, $P < 0.05$ vs. CTR.

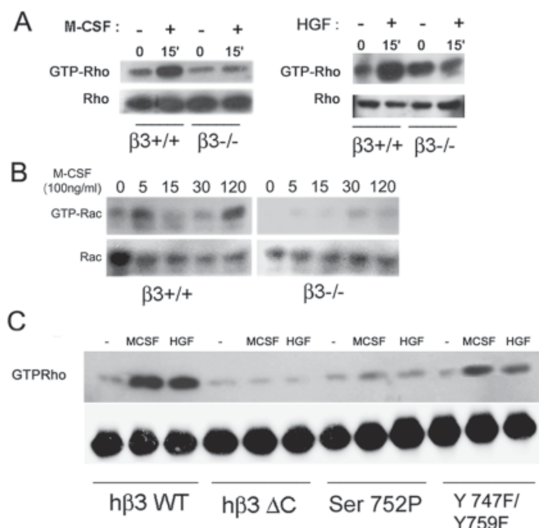

Figure 7. **Growth factor activation of Rho GTPases requires functional β3 cytoplasmic domain.** (A) OCs were exposed to HGF or M-CSF for 15 min, and Rho activation was assessed by pull-down binding assay. $β3^{+/+}$ OCs respond to both growth factors with a sixfold increase in GTP-bound Rho, whereas $β3^{-/-}$ OCs do not respond to either growth factor. Immunoblot for total Rho in cell lysates is shown below. (B) $β3^{+/+}$ and $β3^{-/-}$ cells were stimulated with M-CSF for the indicated times, and Rac activation was assessed by pull-down binding assay. As a loading control, a fraction of the cell lysates was immunoblotted with the Rac mAb. Rac activation occurs only in $β3^{+/+}$ OCs, in a biphasic manner. (C) The experiment described in A was repeated with $β3^{-/-}$ OCs transfected with the indicated β3 constructs. Whereas β3 WT– and β3 $Y^{747}F/Y^{759}F$–bearing OCs respond like their $β3^{+/+}$ counterparts, no such induction occurs in those expressing β3-ΔC or $S^{752}P$.

As substrate recognition initiates lamellipodia extension and directed cell movement, we next assessed the migration of pre-OCs transduced with the various β3 constructs, using a transwell assay in which the lower surface of the membrane was coated with OPN (Fig. 6 B). Pre-OC migration toward OPN is impaired in the absence of the β3 cytoplasmic domain or with the $S^{752}P$ mutation, mimicking the behavior of $β3^{-/-}$ cells (unpublished data). Moreover, AP5, HGF, and M-CSF all fail to enhance the directed mobility of β3-ΔC and $S^{752}P$ mutant cells. Once again, similar results were generated using VN as substrate (unpublished data). Thus, these data mirror the defective growth factor–induced integrin activation of β3-ΔC and $S^{752}P$ mutants (Fig. 3 B).

Rho family GTPase activation is impaired in $β3^{-/-}$ OCs

Numerous signal transduction molecules, including Rho GTPases, associate with integrin complexes in adherent cells

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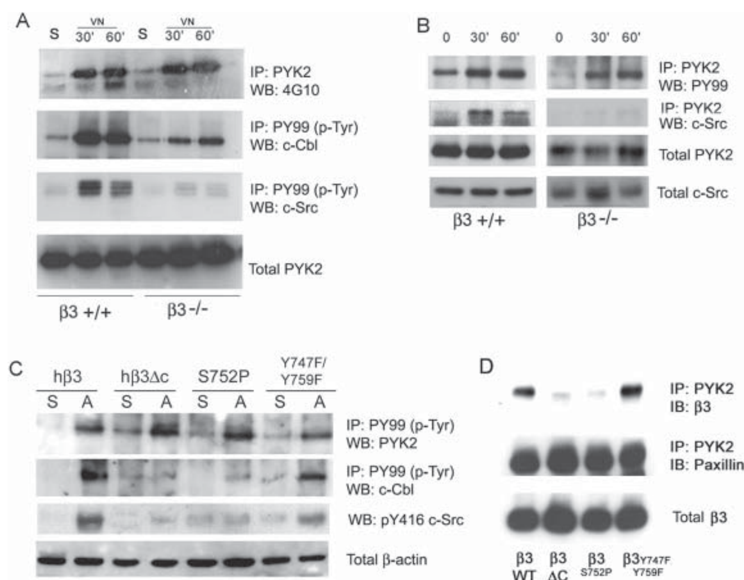

Figure 8. **c-Src and c-Cbl, but not Pyk2, activation is impaired in $\beta_3{}^{-/-}$ OCs.** (A) Serum-starved pre-OCs were lifted and maintained in suspension (S) or replated on VN for 30 or 60 min. Equal amounts of proteins were immunoprecipitated with anti-Pyk2 mAb followed by immunoblot with antiphosphotyrosine mAb 4G10. Aliquots of the same lysate were immunoprecipitated with an antiphosphotyrosine mAb (PY99) followed by c-Src or c-Cbl immunoblot. Pyk2 phosphorylation, although not occurring in suspended cells, is indistinguishable in $\beta3^{-/-}$ and $\beta3^{+/+}$ pre-OCs plated onto VN. In contrast, c-Cbl and c-Src phosphorylation occurs in a β3-dependent manner. (B) Lysates from $\beta3^{+/+}$ or $\beta3^{-/-}$ pre-OCs, adherent with time on VN (30 or 60 min), were subjected to Pyk2 immunoprecipitation followed by c-Src or antiphosphotyrosine (PY99) immunoblot. In the absence of the β3 integrin, Pyk2 does not associate with c-Src. (C) $\beta3^{-/-}$ pre-OCs expressing the indicated β3 mutants were maintained in suspension (S) or were adherent (A) on VN for 30 min. Immunoprecipitation of lysates with antiphosphotyrosine (PY99) preceded immunoblot analysis of Pyk2, c-Cbl, and phospho-Src (Y416). β-Actin is the loading control. c-Src and c-Cbl phosphorylation are arrested in cells expressing β3-ΔC or β3 S^{752} P, but not β3 WT or β3 $Y^{747}F/Y^{759}F$. In all circumstances, Pyk2 phosphorylation remains intact. (D) Adherent OCs expressing the indicated β3 mutants were lysed, immunoprecipitated with Pyk2, and immunoblotted for β3 and paxillin. Association of Pyk2 with β3, but not with paxillin, requires intact β3 cytoplasmic tail and Ser^{752}. Immunoblot for β3 shows similar expression level of the integrin in all mutant cells.

and regulate adhesion-dependent morphological changes (Pavalko et al., 1991; Yamada and Miyamoto, 1995; Clark et al., 1998). Furthermore, distinct aspects of adhesion and migration are controlled by different Rho family members. For example, Rho, Rac, and Cdc42, respectively, regulate the formation of stress fibers, lamellipodia, and filopodia. As $\beta3^{-/-}$ OCs exhibit defective adhesion, spreading, and cytoskeletal rearrangement in response to growth factors, we asked if these phenomena reflect impaired activation of Rho GTPases. To this end, $\beta3^{-/-}$ and $\beta3^{+/+}$ OCs were exposed to HGF or M-CSF, and Rho and Rac activation were assessed by pull-down binding assays. Mirroring the cytoskeletal defects, Rho activation in response to both growth factors is completely arrested in $\beta3^{-/-}$ OCs, compared with the sixfold increase seen in $\beta3^{+/+}$ OCs (Fig. 7 A). Similarly, whereas Rac is activated in a biphasic manner in $\beta3^{+/+}$ OCs exposed to M-CSF, no such activation occurs in $\beta3^{-/-}$ OCs (Fig. 7 B). To determine if integrin-mediated Rho activation is dependent on an intact β3 cytoplasmic domain, we repeated these experiments with $\beta3^{-/-}$ OCs transduced with the various β3 constructs. Whereas β3 WT– and β3 $Y^{747}F/Y^{759}F$–expressing OCs respond to growth factor with an increase in GTP-bound Rho, no induction occurs in those expressing β3-ΔC or β3 $S^{752}P$ (Fig. 7 C).

Phosphorylation of c-Src and c-Cbl, but not Pyk2, is impaired in $\beta3^{-/-}$ OCs

The data presented thus far establish that the β3 integrin plays an essential role in OC cytoskeletal function, a process mediated at least in part by Rho family GTPases. These observations prompted us to examine the more proximal events thought to mediate αvβ3 signal transduction. Pyk2, believed to be central to the mechanisms by which OCs resorb bone, is activated when these cells interact with αvβ3 ligands (Duong et al., 2000; Sanjay et al., 2001). Surprisingly, however, Pyk2 activation, as manifest by its phosphorylation, is indistinguishable in $\beta3^{+/+}$ and $\beta3^{-/-}$ pre-OCs plated on VN for 30 and 60 min (Fig. 8 A, top). In contrast, c-Src phosphorylation occurs in a β3-dependent manner, and c-Cbl phosphorylation is markedly reduced in the absence of the integrin (Fig. 8 A). In keeping with this observation, Pyk2 and c-Src coprecipitate in VN-adherent $\beta3^{+/+}$ cells but not those lacking the β3 integrin (Fig. 8 B). Furthermore, $\beta3^{-/-}$ pre-OCs expressing all β3 constructs show phosphorylation of Pyk2 upon adhesion, whereas c-Src and c-Cbl activation is defective in β3-ΔC and $S^{752}P$ (Fig. 8 C). Pfaff and Jurdic (2001) have recently shown direct interaction of both Pyk2 and paxillin, a structural component of podosomes, with the COOH-terminal region of the integrin β3 tail. Interestingly, we find that binding of Pyk2 to paxillin is identical in all β3 mutants, but its ability to bind the β3 receptor itself is dependent on an intact cytoplasmic tail and S^{752} (Fig. 8 D).

Our data show that Pyk2 activation requires cell–matrix recognition but does not depend upon αvβ3 status. Previous studies have shown that Pyk2 phosphorylation is calcium dependent (Sanjay et al., 2001), and we find that in $\beta3^{+/+}$ and $\beta3^{-/-}$ pre-OCs, the intracellular chelator, BAPTA, eliminates Pyk2 activation (Fig. 9 A). To determine if another OPN receptor or integrin might be responsible for Pyk2 phosphorylation, we assessed this parameter in cells deleted of CD44, which has been shown to mediate monocyte attachment to OPN (Weber et al., 1996), or in cells lacking the integrin α2β1, another adhesive receptor expressed on OCs (unpublished data). Attachment to OPN leads to Pyk2 phosphorylation and to an increase in its association with paxillin in all cells tested (Fig. 9 B). To find the receptor mediating Pyk2 phosphorylation in response to cell adhesion, we turned to the β2 integrin, which is expressed in pre-OCs (Fig. S1) and is known to be a plastic receptor (Hirano et al., 2002). Thus, $\beta3^{+/+}$, $\beta3^{-/-}$, and $\beta2^{-/-}$ pre-OCs were maintained in

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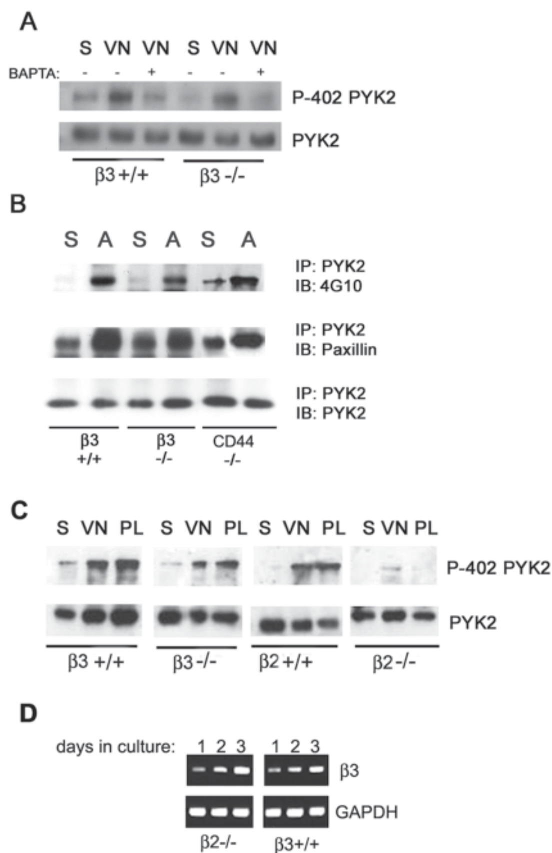

Figure 9. **Pyk2 activation in pre-OCs is dependent upon intracellular calcium and β2 integrin.** (A) $\beta3^{-/-}$ and $\beta3^{+/+}$ pre-OCs were preincubated with the calcium chelator BAPTA before plating on VN for 30 min. Cells were lysed and analyzed by Western blot for total Pyk2 and its Y^{402}-phosphorylated species. BAPTA inhibits Pyk2 phosphorylation in both cell types. (B) $\beta3^{+/+}$, $\beta3^{-/-}$, and $CD44^{-/-}$ pre-OCs were lifted or replated onto OPN for 30 min, and lysates were immunoprecipitated for Pyk2 and analyzed by Western blot for phosphotyrosine (4G10) and paxillin. Cell adhesion leads to Pyk2 phosphorylation and increase in association with paxillin in all cells tested. (C) $\beta3^{+/+}$, $\beta3^{-/-}$, and $\beta2^{-/-}$ pre-OCs were maintained in suspension or plated on VN or plastic (PL) for 30 min. Adhesion on both VN and plastic induced $Pyk2^{Y402}$ phosphorylation in $\beta3^{+/+}$ and $\beta3^{-/-}$ pre-OCs. In contrast, Pyk2 phosphorylation on either VN or plastic is completely abrogated in cells lacking the $\beta2^{-/-}$ integrin. (D) RT-PCR for β3 integrin in $\beta2^{-/-}$ and $\beta2^{+/+}$ pre-OCs cultured for 1, 2, and 3 d with RANKL and M-CSF.

suspension or plated on VN or uncoated plastic for 30 min (Fig. 9 C). Plastic, like VN, induces Pyk2 phosphorylation in wild-type pre-OCs and those lacking αvβ3. However, Pyk2 phosphorylation of $\beta2^{-/-}$ pre-OCs is completely abrogated when these mutant cells are plated on plastic and is barely detectable on VN, an observation that does not reflect diminished β3 integrin expression (Fig. 9 D). Furthermore, these findings do not result from altered Pyk2 levels in $\beta2^{-/-}$ OCs (Fig. 9 C) or differing distribution (unpublished data). Thus, β2 integrin, but not β3 integrin or CD44, mediates Pyk2 activation in pre-OCs.

Discussion

Marrow macrophages derived from $\beta3^{-/-}$ mice, placed in standard osteoclastogenic conditions, fail to become fully differentiated OCs and resorb mineralized matrix poorly (Faccio et al., 2003). On the other hand, culture of these cells in high concentrations of M-CSF, as undertaken in this work, completely rescues the differentiation of these integrin-deficient OCs as manifest by their histological appearance and expression of osteoclastogenic markers. It is surprising, therefore, that despite the normal appearance in high dose M-CSF, $\beta3^{-/-}$ OCs remain defective resorbers. To define the mechanisms responsible for the continued failure of $\beta3^{-/-}$ OCs, generated in high M-CSF, to resorb bone, we analyzed the differences in cytoskeletal organization and relevant intracellular signaling molecules in OCs with and without functional β3 integrins.

αvβ3, in OCs, exists in two conformational states, which are differentially distributed on the cell surface (Faccio et al., 2002). In its basal condition, the receptor localizes in the sealing zone and podosomes, while the activated integrin is principally associated with motile areas of the membrane. The extracellular components of αvβ3 modulating its activation state are in hand (Beglova et al., 2002), and those in the cytoplasmic domain, which respond to growth factor stimulation and thus mediate inside-out signaling, have been partially identified (Takagi et al., 2002; Vinogradova et al., 2002). To further address this issue, we turned to a system of retroviral transduction, which previously permitted us to express various human β3 integrin mutants in OCs and their precursors (Feng et al., 2001).

Our first exercise established that the β3 cytoplasmic domain is essential for appropriate distribution of the integrin to cytoskeletal structures such as podosomes and lamellipodia. Mirroring their effect on OC spreading and matrix resorption, β3 mutants, lacking the cytoplasmic domain or bearing $S^{752}P$, distribute abnormally in OCs residing on glass and dentin, failing to colocalize with F-actin. This observation prompted us to ask if the same components of the β3 integrin are involved in modulating the conformational state of the intact heterodimer.

Antibodies recognizing the activated conformation bind the LIBS in the NH_2 terminus of integrin heterodimers (Bodeau et al., 2001). In high calcium buffer, the anti-LIBS mAb AP5 recognizes activated, high-affinity ligand-binding αvβ3 integrin. Importantly, in low calcium buffer, AP5 binds to all αvβ3 and forces all receptors into the activated conformation. This AP5-induced conformational change of αvβ3 involves a direct effect on the external domain of the integrin and does not require the β3 cytoplasmic domain. On the other hand, the heterodimer's biological activity requires the cytoplasmic tail. Thus, $\beta3^{-/-}$ OCs bearing β3-ΔC or β3 $S^{752}P$ have decreased adhesive and migratory capabilities, suggesting defective outside-in signaling. In contrast to the direct effect of AP5 in changing the external conformation of β3, HGF and M-CSF modulate adhesion and spreading of mature OCs by activating the αvβ3 integrin via the β3 cytoplasmic domain by inside-out signaling (Insogna et al., 1997; Teti et al., 1998; Faccio et al., 2002).

Active OCs form a stable ring of actin, which delineates the ruffled membrane where the resorptive process takes place. Our previous observations, showing abnormal actin rings in β3-null OCs generated in low dose M-CSF, indicate that β3 contributes to the formation of this structure.

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We have also found that αvβ3 and M-CSF cooperate during osteoclastogenesis (Faccio et al., 2003). In this study, we find that $\beta3^{-/-}$ OCs, or those expressing the human mutation $S^{752}P$, exhibit normal actin distribution when generated in high dose M-CSF, indicating that M-CSF can compensate for lack of αvβ3 in actin ring formation.

Podosomes, found in adherent OCs, are rosette-like structures containing αvβ3 around an actin core (Marchisio et al., 1988). In contrast to the relatively static actin ring, podosomes are dynamic, rapidly redistributing under the influence of extracellular stimuli such as HGF and M-CSF (Insogna et al., 1997; Teti et al., 1998; Faccio et al., 2002). Here we show that β3 integrin is absolutely required for dynamic changes in the actin cytoskeleton in response to growth factors or cell attachment. β3-ΔC or β3 $S^{752}P$ mutants fail to form lamellipodia when plated on glass (Fig. 4 A) or on dentin (Fig. 4 B). In agreement with these observations, α-actinin, which links actin filaments directly to integrin receptors (Pavalko et al., 1991; Otey et al., 1993), fails to enter the Triton X-100 soluble fraction of $\beta3^{-/-}$ pre-Ocs, or β3-ΔC and β3 $S^{752}P$ mutants, in response to growth factors. In other cell types, redistribution of α-actinin, from focal adhesions to the Triton X-100 soluble fraction, is associated with loss of close apposition of cell membrane to the extracellular matrix, consistent with enhanced motility (Greenwood et al., 2000).

Modulation of the OC cytoskeleton is controlled by Rho GTPases. For example, dominant negative Rho arrests podosome organization, OC motility, and bone resorption (Chellaiah et al., 2000), and Rac inhibition decreases the resorptive activity of OCs (Razzouk et al., 1999). The mechanisms of Rho and Rac activation in OCs are poorly defined. We find that the defect in migration and lamellipodia formation in β3 $S^{752}P$ OCs, in response to growth factors, is associated with failure to activate Rho and Rac.

Interestingly, OCs bearing β3 $Y^{747}F/Y^{759}F$ are indistinguishable from wild-type cells in their appearance and resorptive capabilities, but adhesiveness to OPN is moderately decreased. Recently, phosphorylation levels of the β3 Y747 and 759 have been correlated with strength of binding during adhesion (Boettiger et al., 2001). It is possible that in OCs, these tyrosines are required for stable and strong adhesion, but not for efficient migration. As motility is requisite for bone resorption, the $Y^{747}F/Y^{759}F$ mutation does not compromise the physiological function of these cells.

The ability of M-CSF to induce OC spreading and actin reorganization depends on c-Src expression (Insogna et al., 1997). Sanjay et al. (2001) have shown that upon adhesion, αvβ3 forms a complex with Pyk2 and c-Src that, in turn, recruits c-Cbl, resulting in podosome assembly. This model holds that c-Cbl binds to Tyr [416] in the c-Src kinase domain, which down-regulates both Src kinase activity and integrin-mediated adhesion, prompting podosome detachment and subsequent disassembly. Consistent with this hypothesis, we find that activation of c-Src and c-Cbl, in response to VN adherence, is abrogated in $\beta3^{-/-}$ OCs and in β3-ΔC and β3 $S^{752}P$ mutants, thereby decreasing podosome turnover and, consequently, OC adhesion and migration.

Our data, however, stand in contrast to the conclusions of Sanjay et al. (2001) and Nakamura et al. (2001), who claim that αvβ3 is essential for Pyk2 phosphorylation. We believe this discrepancy may reflect the fact that we directly assessed Pyk2 phosphorylation in authentic pre-OCs, deleted of the αvβ3 receptor. Two possibilities present themselves as to how OCs lacking αvβ3 phosphorylate Pyk-2. First, Pyk2 activation is calcium dependent (Sanjay et al., 2001). In this regard, the intracellular calcium chelator, BAPTA, blunts Pyk2 autophosphorylation in $\beta3^{+/+}$ and $\beta3^{-/-}$ pre-OCs. Second, other adhesive receptors could compensate for the lack of β3 and mediate Pyk2 phosphorylation. Pyk2 activation occurs equally in cells lacking α2β1 integrin or CD44, another receptor for OPN, but not in $\beta2^{-/-}$ pre-OCs. Thus, although it is formally possible that α2β1, CD44, and β3 compensate for each other in signaling to Pyk2, the weak Pyk2 phosphorylation detected in $\beta2^{-/-}$ cells suggests that the β2 integrin is dominant in this process. In support of this posture, uncommitted macrophages, which have yet to express αvβ3, activate Pyk2 by β2 integrin ligation (Duong and Rodan, 2000), and we find that the same is true in $\beta3^{-/-}$ pre-OCs. Despite having the β2 integrin, $\beta3^{-/-}$ pre-OCs generated in high dose M-CSF do not retain a macrophage phenotype, as they express markers of committed OCs. As the β2 integrin is not present in fully mature resorptive OCs (Athanasou and Quinn, 1990), in this circumstance, αvβ3 may be the Pyk2 activating receptor.

Pyk2, independent of its phosphorylation status, is associated with paxillin, and this association is increased in adherent cells. Pyk2, however, fails to be recruited to the β3 com-

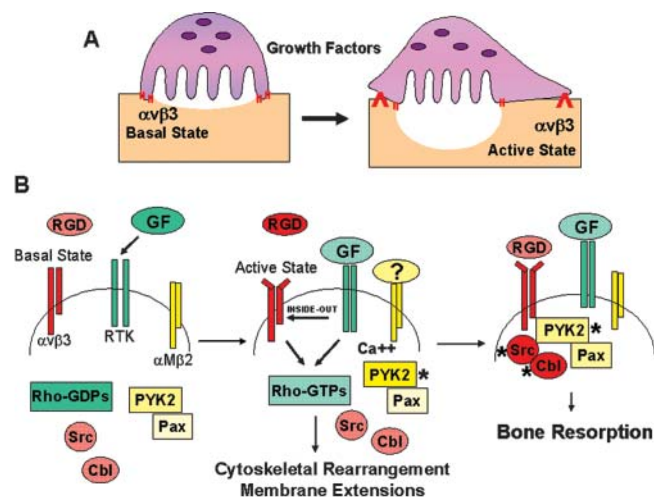

Figure 10. **Model of OC αvβ3 integrin activation and signal transduction.** (A) αvβ3 in its basal conformation (||) exists within the actin ring. Resorption is stimulated by growth factors and requires activated αvβ3 (Λ), which localizes in newly formed membrane extensions. (B) Binding of a growth factor (GF) to its receptor changes the conformation of the external domain of αvβ3 from the basal to the activated state. The GF receptor tyrosine kinase (RTK) and αvβ3 collaboratively induce cytoskeletal rearrangements via activation of Rho family proteins from their GDP-bound to GTP-bound forms. Pyk2 is phosphorylated (*) by αMβ2 binding an unknown ligand (?) or increased intracellular calcium. Upon ligand (RGD) occupancy of αvβ3, phosphorylated (*) Pyk2 forms a complex at the β3 cytoplasmic domain with phosphorylated c-Src and c-Cbl. Whereas Pyk2 is constitutively associated with paxillin, formation of the Pyk2–c-Src–c-Cbl adhesive complex, and thus efficient bone resorption, requires αvβ3.

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plex in adherent cells carrying β3-ΔC or $S^{752}P$. It is possible that the failure of c-Src and c-Cbl to be activated in these mutants reflects the inability of Pyk2 to bind the integrin.

We propose, therefore, that in OCs, cytokines stimulate the formation of new membrane extensions that contain activated αvβ3 (Fig. 10 A). These cytoskeletal rearrangements are under the control of Rho family GTPases and require functional αvβ3 (Fig. 10 B). Upon αvβ3 occupancy, phosphorylated Pyk2, an event independent of the integrin, forms a complex at the β3 cytoplasmic domain with phosphorylated c-Src and c-Cbl (Fig. 10 B). In the absence of functional αvβ3, Pyk2 may be activated by other means, such as αMβ2 or increased calcium. These alternative means of activating Pyk2 permit its association with paxillin, but the Pyk2–c-Src–c-Cbl adhesive complex fails to form, resulting in poorly resorptive OCs.

Materials and methods

Murine OCs

BMMs were isolated from long bones of 4- to 8-wk-old mice by culturing whole marrow for 3 d in α-MEM containing 10% heat-inactivated FBS and 1:10 CMG supernatant as a source of M-CSF (Faccio et al., 2003).

Infection of BMMs

BMMs were transduced with virus containing vectors that encode for several β3 integrin mutants (Feng et al., 2001), in the presence of 1:10 CMG supernatant and 8 μg/ml polybrene (Sigma-Aldrich), without antibiotic selection. Cells were cultured for an additional 2–3 d before analysis of integrin expression or osteoclastogenesis.

Flow cytometry

Pre-OCs expressing the different mutants were lifted with Trypsin/EDTA (Sigma-Aldrich) and washed in a calcium-free buffer based on HBSS. Pretreated cells were incubated with HGF or M-CSF in α-MEM supplemented with 0.5% BSA for 30 min at 37°C; control cells were incubated with medium alone. After incubation, cells were washed twice and incubated with the mAb AP5 (50 μg/ml) in high calcium buffer, which recognizes the activated β3 integrin subunit. Binding of AP5 in HBSS calcium-free buffer served as positive control, identifying all β3 on the cell surface. Cells were then incubated with FITC-conjugated secondary Ab, as previously described (Faccio et al., 2002).

Immunofluorescence

$\beta3^{+/+}$ or $\beta3^{-/-}$ BMMs, transduced with the indicated mutants, were plated on dentin slices or glass coverslips under osteoclastogenic conditions for 4 d. For some experiments (Figs. 3 and 4), after 4 d in culture, cells were treated with HGF (50 ng/ml) or M-CSF (100 ng/ml), or media + 0.5% BSA as control, for 30 min at 37°C, and then fixed and stained as previously described (Faccio et al., 2002) and observed with a confocal microscope.

Adhesion and migration assays

Adhesion and haptotactic migration assays were performed using pre-OCs expressing the different β3 mutants plated respectively onto 96-well plates or transwell filters, 8-μm pore size (Costar), coated with 10 μg/ml human OPN.

For both experiments, cells were preactivated with growth factors or AP5 for 30 min in suspension and then plated, and adherent cells were stained with crystal violet. For migration assay, cells that migrated to the lower side were viewed at 300× magnification and counted. Results represent the averages from 15 fields ± SEM of a representative experiment.

Western blot analysis

BMMs were cultured for 3 d in the presence of 100 ng/ml RANKL and 100 ng/ml M-CSF and starved overnight in the presence of 2% serum. Cells were lifted and replated onto the indicated matrix proteins for 30 or 60 min. In some experiments, adherent OCs were starved and restimulated with 100 ng/ml M-CSF or 50 ng/ml HGF. Cells were lysed in RIPA (Faccio et al., 2003), or in TNE (Lakkakorpi et al., 1999) for coimmunoprecipitation of c-Src and Pyk2. Precleared lysates were immunoprecipitated with 2 μg anti-Pyk2 polyclonal antibodies (Biosource International, CA), 2 μg anti-β3 mAb (clone 7G2), or 2 μg antiphosphotyrosines (PY99) for 1 h at 4°C followed by overnight incubation with protein A/G–Sepharose beads at 4°C (Santa Cruz Biotechnology, Inc.) and then analysis by SDS-PAGE and immunoblotting.

Rho and Rac assay

Pre-OCs were lysed in a buffer containing 50 mM Tris/HCl, pH 7.5, 1 mM EDTA, 500 mM NaCl, 10 mM $MgCl_2$, 1% (vol/vol) Triton X-100, and protease inhibitors (4 μg/ml leupeptin and 30 μg/ml PMSF). Lysates were incubated with glutathione–agarose beads (Sigma-Aldrich) coupled with bacterially expressed GST–RBD fusion protein for Rho pull down or GST–PAK1 for Rac pull down (Ren et al., 1999) at 4°C for 45 min. Bound proteins were analyzed by SDS-PAGE followed by immunoblotting against RhoA (Santa Cruz Biotechnology, Inc.) or Rac1 (Upstate Biotechnology).

Online supplemental material

The supplemental material (Figs. S1 and S2) is available at http://www.jcb.org/cgi/content/full/jcb.200212082/DC1. Fig. S1 shows the expression levels of c-Fms, α2β1, and β2 in BMMs and pre-OCs. Fig. S2 shows the localization of viniculin, talin, and β3 integrin in the podosomes.

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