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Osteoclast motility: putting the brakes on bone resorption

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Abstract

As the skeleton ages, the balanced formation and resorption of normal bone remodeling is lost, and bone loss predominates. The osteoclast is the specialized cell that is responsible for bone resorption. It is a highly polarized cell that must adhere to the bone surface and migrate along it while resorbing, and cytoskeletal reorganization is critical. Podosomes, highly dynamic actin structures, mediate osteoclast motility. Resorbing osteoclasts form a related actin complex, the sealing zone, which provides the boundary for the resorptive microenvironment. Similar to podosomes, the sealing zone rearranges itself to allow continuous resorption while the cell is moving. The major adhesive protein controlling the cytoskeleton is $\alpha v \beta 3$ integrin, which collaborates with the growth factor M-CSF and the ITAM receptor DAP12. In this review, we discuss the signaling complexes assembled by these molecules at the membrane, and their downstream mediators that control OC motility and function via the cytoskeleton.

Keywords

$\alpha v \beta 3$ integrin; M-CSF; podosome; migration

Skeletal Aging

Maintenance of skeletal integrity is an important part of healthy aging. In both women and men, peak bone mass is attained in early adulthood, and significant declines occur as levels of sex hormones drop, leading to osteoporosis. In the mature skeleton, the process of remodeling constantly replaces bone elements, with an average turnover interval of about 10 years. The basic multicellular unit (BMU) consists of the coupled activity of osteoclasts (OCs), which remove bone, and osteoblasts (OBs), which lay down the new organic matrix on which mineralization occurs. Bone mass is stable when the amount of bone removed by OCs is evenly balanced by osteoblastic bone formation. Bones have a dense outer shell, the cortex, and an inner network of trabeculae composed of a dense network of rods and plates, which impart structural stability, while allowing for needed flexibility. Over time, both cortical and trabecular bone is remodeled. In osteoporosis, bone resorption outpaces bone formation, resulting in net bone loss, particularly in the trabeculae-rich bone of vertebrae and femoral neck, which are common sites of fragility fracture.

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OC differentiation

The only cell capable of removing both the organic and inorganic matrices of bone is the OC, a multinucleated cell derived from fusion of bone marrow cells in the monocyte/macrophage lineage (Ikeda, et al., 1998). Like other monocyte/macrophages, they require the growth factor M-CSF to interact with its receptor c-Fms, a receptor tyrosine kinase (RTK), which provides proliferative signals to the precursors as well as survival signals for mature cells (Ross and Teitelbaum, 2005). Differentiation to the OC lineage also requires Receptor Activator of NF- κ B Ligand (RANKL), a TNF family cytokine that binds to the receptor RANK (Lacey, et al., 1998; Yasuda, et al., 1998). RANK is related to other TNF receptors, and has a long cytoplasmic tail that complexes with signaling modules activating NF- κ B, JNK/AP1, Ca/calmodulin, c-Src and PI3K pathways (Novack and Teitelbaum, 2008). Signaling via RANK requires costimulation via one of 2 ITAM-containing receptors, DAP12 and Fc γ (Koga, et al., 2004; Mocsai, et al., 2004). For differentiation, cooperative activation of the NF- κ B, JNK/AP1, and Ca/calmodulin pathways leads to activation of the transcription factor NFATc1, a primary driver of expression of many genes required for OC function, including cathepsin K, β 3 integrin, and calcitonin receptor (Ikeda, et al., 2004). NF- κ B activity is one of the earliest signals following RANKL stimulation, and is required for precursor survival during commitment to the OC lineage (Vaira, et al., 2008a) as well as for full differentiation (Vaira, et al., 2008b). OCs can be differentiated in vitro from bone-marrow derived macrophages by treatment with soluble M-CSF and RANKL. In vivo, OBs and their precursors appear to be the most important source of both M-CSF and RANKL for osteoclastogenesis (Tsurukai, et al., 2000).

Another necessary feature of OC differentiation is fusion of mononuclear precursors to form the mature polykaryon. Transmembrane proteins DC-STAMP and Atp6v0d2, a subunit of the vacuolar ATPase, are required for OC multinucleation, and both are transcriptionally regulated by NFATc1 (Kim, et al., 2008; Yagi, et al., 2005; Yagi, et al., 2007). Mononuclear OCs lacking DC-STAMP or Atp6v0d2 have decreased bone-resorbing capacity, despite normal expression of markers of differentiation.

Resorptive machinery of an active OC

Mature OCs polarize when attached to bone, which occurs via the α v β 3 integrin that recognizes matrix proteins such as bone sialoprotein and osteopontin. The integrin and an associated cytoskeletal complex (discussed below) form a sealing zone, a ring-shaped boundary where the OC membrane is very closely apposed to the bone surface, defining an isolated extracellular space between the OC and the bone surface. In fact, at least in vitro, individual OCs can form more than one sealing zone. Bone resorption occurs in the space between the bone and OC defined by the sealing zone, known as the resorption lacuna. A specialized resorptive organelle, the ruffled membrane, is formed by fusion of secretory vesicles into the plasma membrane within the sealing zone. This directional vesicular trafficking occurs along microtubules, and then microfilaments, mediated by the small GTPases Rab7, Rab3D and Rac1 (Coxon and Taylor, 2008; Sun, et al., 2005). The vesicles contain the H⁺/ATPase needed for secretion of acid, and the CIC-7 chloride channel, which maintains electroneutrality across the OC membrane. In order to accomplish acidification, the OC also requires activity of a HCO₃⁻/Cl⁻ exchanger on the basolateral membrane and cytoplasmic carbonic anhydrase, which generates HCO₃⁻ and H⁺ from CO₂ and H₂O. Acidification of the lacunar space due to secretion of HCl dissolves the mineral hydroxyapatite, which precedes enzymatic degradation of collagen. Abnormalities of components of the acidification pathway, such as the H⁺/ATPase (Frattoni, et al., 2000) or CIC-7 (Kornak, et al., 2001) cause osteopetrosis, a rare inherited disease in which the bones become very dense and fracture easily. Matrix proteases, particularly cathepsin K, are also

present in the vesicles transported to the ruffled membrane (Saftig, et al., 1998). Inactivating mutations of cathepsin K in humans cause pyknodysostosis, a disorder characterized by increased bone mass, dwarfism and facial dysmorphism (Hunt, et al., 1998). Thus, a combination of acidification and enzymatic digestion of organic matrix components cooperate to fully remove bone within this well-defined area (Novack and Teitelbaum, 2008).

Podosomes: organizing principle of the OC cytoskeleton

The primary component of the cytoskeleton is F-actin, and the OC has a distinct actin complex known as the podosome which mediates its attachment to the bone matrix (Destaing, et al., 2003). Podosomes contain an F-actin core that also concentrates actin regulatory proteins including cortactin, Wiskott Aldrich Syndrome Protein (WASP), WASP Interacting Protein (WIP), Arp2/3, and gelsolin, as well as CD44, another adhesion receptor. Surrounding the core are adhesion-associated proteins such as integrins, vinculin, paxillin, and talin (Linder and Deschenes, 2007; Linder and Aepfelbacher, 2003; Marchisio, et al., 1988; Pfaff and Jurdic, 2001; Zamboni-Zallone, et al., 1989). Co-staining of OCs for actin and $\alpha\beta3$ integrin or vinculin shows a characteristic “donut” of the latter complex of adhesive proteins enclosing a dense actin core. The area surrounding the actin core is also rich in kinases such as c-Src and Pyk2, and the small GTPases Rho, Rac, and cdc42. Also surrounding the dense actin core is a loose network of F-actin cables known as the actin cloud. Although both the actin core and the actin clouds are essential for proper podosome organization and bone resorption, they appear to be independent structures. OCs lacking the kinase c-Src do not have an actin cloud, while those lacking WIP have no actin core (Chabadel, et al., 2007; Destaing, et al., 2008). Ultimately, both molecules are essential for podosome formation, since both c-Src- and WIP-deficient OCs have resorptive defects.

In vivo imaging studies using preosteoclasts expressing green fluorescent protein-actin plated on glass have been useful in studying podosome dynamics. In OC precursors, podosomes randomly distribute throughout the cell, then coalesce into simple clusters and rings as differentiation proceeds. Continuous addition of new podosomes at the outer edge of each ring, with suppression of podosome formation within rings finally generates a single podosome belt at the periphery of mature OCs (Destaing, et al., 2003). Interestingly, the podosome belt is surrounded by an actin cloud, which interconnects all podosome cores (Saltel, et al., 2008). Although the podosome belt appears to be a stable structure, individual podosomes are short lived, with an average lifespan of 2-12 min. Photobleaching experiments show that the F-actin within each podosome is even more dynamic, with recovery in 1 min, suggesting that the podosome belt is also a site of continuous actin polymerization.

How is it that the podosome belt, comprised of dynamic and short-lived actin structures, does not dissolve? It is believed that podosome belts are stabilized by a network of microtubules (MTs), although the exact molecular interactions between F-actin and MTs have not been elucidated (Jurdic, et al., 2006). Indeed, treatment with nocodazole (an MT depolymerizing agent), while not affecting podosome formation per se, controls the transition between podosome clusters and rings into peripheral belts. During this transition, tubulin, the subunit of MTs, accumulates a specific post-translational modification, acetylation. The specific function of this acetylation is still unknown, but it is believed to play an important role in MT stabilization because only the pool of MTs associated with the stable podosome belt is acetylated. Further evidence of the functional importance of MT acetylation comes from the study of active OCs (Saltel, et al., 2008). When plated on bone (or on surfaces mimicking bone such as apatite collagen complex) that can be resorbed, OCs do not form single podosomes or the peripheral podosome belts typical of cells plated on

glass. Instead these resorptive cells organize an inner actin ring which is the functional unit of the OC membrane at the sealing zone (Luxenburg, et al., 2007) (Figure 1, left). Although the actin ring in the sealing zone is thicker than in the podosome belt, the molecular components (eg. $\alpha\text{v}\beta 3$, vinculin, c-Src, Pyk2, small GTPases) are the same. Like the podosome belt, the sealing zone is associated with acetylated MTs, suggesting that its stability is also MT dependent.

MT stability is also regulated by the small GTPase Rho. Indeed, Rho inhibition by the bacterial exoenzyme C3 induces formation of stable and highly acetylated MTs, making the podosome belt more resistant to dissolution by nocodazole (Destaing, et al., 2005). Colocalizing with MTs is a complex Rho itself, mDIA2, a Rho effector, and HDAC6, a cytosolic histone deacetylase which deacetylates MTs (Matsuyama, et al., 2002). The interaction between mDIA2 and HDAC6 increases the deacetylase activity of the enzyme. Thus it appears that Rho modulates the stability of the MTs, by decreasing acetylation of tubulin in a mDIA2/HDAC6-dependent manner. During osteoclastogenesis, this pathway is downregulated to allow the acetylation of a subset of MTs which are important for the formation of the podosome belt (on glass) and sealing zone (on bone).

Visualization of the pits formed by OCs plated on bone demonstrates that each OC typically produces a “trail” that is longer than the cell is wide, indicating that migration is part of the resorption process. As the cell moves, it rearranges its cytoskeleton to form new sealing zones and dissolve old ones (Figure 1, right). In fact, a single resorbing OC on bone can have multiple sealing zones. Although the exact signals leading to sealing zone dissolution and reassembly remain undefined, it is likely that dynamic regulation of MTs by the Rho pathway is central to this process.

M-CSF: a migratory stimulus and actin regulator

In most cells, migration involves directed protrusion at the leading edge through localized polymerization of actin, forming a membrane extension called a lamellipodium. Integrin-mediated adhesion to the substrate stabilizes adhesions, allowing cells to generate tension and the contractile force required for cell movement. Adhesion is then released at the rear of the cell to allow continued forward movement. Migration in OCs is regulated by 2 main pathways, $\alpha\text{v}\beta 3$ integrin, which mediates substrate attachment, and M-CSF, which is a potent chemotactic stimulus for these cells. Re-addition of M-CSF to quiescent macrophages and OCs stimulates rapid cytoskeletal reorganization, podosome dissolution and lamellipodia formation (Faccio, et al., 2003a; Fuller, et al., 1993). Exposure to the cytokine also increases cell motility within a few minutes, followed by chemotactic migration up a gradient of diffusing cytokine.

M-CSF binds to its receptor c-Fms, a member of the receptor tyrosine kinase (RTK) family and induces receptor autophosphorylation at seven tyrosine residues within the cytoplasmic domain (Pixley and Stanley, 2004; Ross and Teitelbaum, 2005). Several Src homology 2 (SH2) and PTB domain-containing molecules are recruited to the phospho-Tyr residues upon M-CSF binding and initiate cell proliferation, differentiation, and cytoskeletal reorganization. The key members of the signaling complex from c-Fms to the cytoskeleton are c-Src, Syk, PI3K, and Cbl.

By using a chimeric receptor approach consisting of the extracellular domain of the erythropoietin receptor (EpoR) fused to the transmembrane domain and cytoplasmic tail of c-Fms, expressed in macrophages and osteoclasts, critical c-Fms Tyr residues modulating the migratory response and actin dynamics have been identified in these cells (Faccio, et al., 2007). Specific Tyr to Phe mutations confirm that c-Fms Tyr-559 binds c-Src in OCs and other Src Family Kinases (SFKs) in macrophages, while Tyr-721 binds phosphatidylinositol

3-kinase (PI3K). Surprisingly, despite the known role of PI3K in M-CSF-dependent signaling to the cytoskeleton (Grey, et al., 2000; Jones, et al., 2003), the c-Fms Tyr-721 to Phe mutant, while affecting the direct binding of PI3K, does not impair podosome redistribution or the chemotactic response to M-CSF. Indeed, it is Tyr-559 of c-Fms which is required for M-CSF dependent cytoskeletal changes, lamellipodia formation and motility. These data suggest that although PI3K is important for M-CSF-induced macrophage and OC motility, direct binding of PI3K to c-Fms is not necessary.

Co-immunoprecipitation studies demonstrate that a ternary complex between c-Src, Cbl and PI3K forms at Tyr559 (Faccio, et al., 2007). Upon M-CSF stimulation, c-Src becomes phosphorylated and binds to c-Fms-Tyr559 via its SH2 motif. c-Src also binds the proline rich region of Cbl with its SH3 domain and phosphorylates it. Cbl then recruits PI3K to activate the downstream pathways leading to actin reorganization. Although the c-Src/Cbl/PI3K complex is anchored to c-Fms-Tyr⁵⁵⁹, it does not form in cells expressing a mutant c-Fms receptor with the Tyr⁵⁵⁹ as the sole intact tyrosine. Instead, this complex requires stabilization via secondary binding to Tyr⁶⁹⁷ and Tyr⁷²¹ of c-Fms. In concert with the critical role of the c-Src/Cbl/PI3K complex, cooperation between c-Fms Tyr⁵⁵⁹, Tyr⁶⁹⁷ and Tyr⁷²¹ is required to restore the full chemotactic response to M-CSF (Figure 2). This set of interactions eventuates in Vav3/Rac activation and thus cytoskeletal reorganization (see below for details).

$\alpha v\beta 3$ integrin: linking the OC to bone

Dynamic changes in the osteoclast cytoskeleton associated with the bone resorptive process are controlled by $\alpha v\beta 3$ integrin (Faccio, et al., 2003a). Deletion of $\beta 3$ in mice leads to a progressive increase in bone mass, due to failure of resorption (McHugh, et al., 2000). When differentiated in vitro, $\beta 3^{-/-}$ osteoclasts do not spread or form a podosome belt when plated in physiological amounts of RANKL and M-CSF (Feng, et al., 2001). Confirming their attenuated resorptive activity, $\beta 3^{-/-}$ osteoclasts generate fewer and shallower resorptive lacunae on dentin slices than do their wildtype counterparts. Furthermore, absence of $\beta 3$ integrin also impairs motility towards M-CSF (Faccio, et al., 2003a). Consequently, anti- $\alpha v\beta 3$ antibodies (Horton, et al., 1991), and peptides and proteins containing the arg-gly-aspartate (RGD) motif recognized by the integrin (Fisher, et al., 1993; Horton, et al., 1991) inhibit bone degradation, in vitro, and are now under investigation for their use in vivo.

Similar to other integrin receptors, $\alpha v\beta 3$ exists in two configurations, which confer either low affinity or high affinity towards RGD motif containing proteins in the bone matrix, such as osteopontin. In the default low affinity state, the extracellular domain of the receptor assumes a bent conformation which is maintained by interactions between the cytoplasmic tails of the α and β subunits (Takagi, et al., 2002). Separation of the two cytoplasmic tails causes the extracellular domain to straighten, leading to an open configuration that allows efficient ligand binding, thereby increasing its affinity. The process of conformational change is known as integrin activation, and it can occur by two distinct mechanisms known as outside-in and inside-out activation.

In outside-in activation, ligand initially binds to $\alpha v\beta 3$ in its low affinity state causing conformational changes that enhance the affinity of the integrin receptor (Qin, et al., 2004). Artificially induced outside-in activation can also be caused by extracellular chemicals or monoclonal antibodies prior to ligand binding (Honda, et al., 1995).

In contrast, inside-out activation is an indirect process in which intracellular signals driven by another receptor, typically that of a cytokine or growth factor, affects the intracellular domain of the integrin. In the OC, M-CSF targets the $\beta 3$ subunit cytoplasmic domain, and alters the conformation of $\alpha v\beta 3$ to its high affinity, ligand-binding state (Faccio, et al., 2002;

Faccio, et al., 2003a). M-CSF also induces a stable interaction between c-Fms and $\alpha\beta3$ in these cells (Elsegood, et al., 2006). Additionally, defective c-Fms prevents OC spreading on $\alpha\beta3$ -ligand coated surfaces (Faccio, et al., 2007). The consequences of this cytokine-induced, inside-out activation of $\alpha\beta3$ are increased adhesion, motility and stimulation of the resorptive signaling pathway. These experiments support a cooperative model, in which $\alpha\beta3$ and M-CSF signals are both required for optimal bone resorption.

$\alpha\beta3$ integrin and OC motility

$\alpha\beta3$ integrin localizes around the actin core of podosomes in the podosome belt of OCs plated on glass (Figure 3, left). When OCs are stimulated to migrate with M-CSF, their cytoskeleton rapidly reorganizes. Podosome belts dissolve and $\alpha\beta3$ moves to the leading edge of the lamellipodia (Figure 3, right). In resorbing cells on bone, $\alpha\beta3$ integrin is distributed along the inner and outer edge of the ring of actin forming the sealing zone. The cytoplasmic tail of the integrin receptor is required for its localization to the podosomes and lamellipodia. Retroviral expression of various $\beta3$ integrins with cytoplasmic tail mutations in $\beta3$ -deficient OC precursors revealed that Ser⁷⁵² is important for M-CSF-induced cytoskeletal reorganization and membrane protrusion. Mutation of Ser⁷⁵² to Pro prevented OC adhesion, migration, and bone resorption (Faccio, et al., 2003a). This finding is particularly important in light of the human disease called Glanzmann's thrombasthenia, characterized by excessive bleeding, in which the mutation of $\beta3$ Ser⁷⁵² to Pro is causative. Osteopetrosis was associated in one individual with this disease (Yarali, et al., 2003), but most patients have relatively normal bone mass, perhaps due to compensation by $\beta1$ integrins (Horton, et al., 2003).

The $\beta3$ cytoplasmic tail also contains Tyr⁷⁴⁷ and Tyr⁷⁵⁹. While affecting platelet functions, $\beta3$ Tyr^{747/759}Phe mutants expressed in OCs do not have any effect on the appearance and resorptive capabilities of these cells, despite moderately decreased adhesiveness to OPN (Faccio, et al., 2003a). In other cellular contexts, phosphorylation levels of the $\beta3$ Tyr⁷⁴⁷ and Tyr⁷⁵⁹ have been correlated with strength of binding during adhesion (Boettiger, et al., 2001). It is possible that during the dynamic process of migration and resorption in OCs, stable and strong adhesion, such as that supported by phosphorylation of these two Tyr residues, is not required.

$\alpha\beta3$ adhesion complex

Engagement of the $\alpha\beta3$ integrin leads to remodeling of the actin cytoskeleton through the formation and activation of a large "adhesion complex", containing enzymes, adaptors and scaffolding proteins. One major component of this complex in the OC is c-Src. c-Src knockout mice are severely osteopetrotic with OCs that fail to resorb bone (Soriano, et al., 1991). In Src^{-/-} OCs, the peripheral podosome belt is absent and replaced by irregular patches at the cell center, likely due to a decrease in podosome number and/or altered dynamics (Destaing, et al., 2008). Not surprisingly, Src^{-/-} OCs also show impaired adhesion, spreading and migration (Destaing, et al., 2008; Insogna, et al., 1997; Sanjay, et al., 2001).

The similar cytoskeletal phenotypes of c-Src and $\alpha\beta3$ -deficient osteoclasts further indicate a commonality of intracellular signaling between the two molecules. c-Src binds directly to the terminal three amino acids of the $\beta3$ subunit in the context of the platelet integrin $\alpha\text{IIb}\beta3$ (Arias-Salgado, et al., 2003), and the same residues regulate $\alpha\beta3$ -c-Src association in the OC (Zou, et al., 2007). Activation of c-Src in this complex is $\alpha\beta3$ -ligand dependent. Phosphorylation at Tyr⁵²⁷ by C-terminal Src kinase (Csk) induces an intramolecular interaction with the SH2 domains of Src that inhibits Src kinase activity, whereas autophosphorylation at Tyr⁴¹⁶ is required for its activation (Yeatman, 2004). Protein-protein interactions with the SH2 and SH3 domains can also relieve the intramolecular inhibitory

state and serve to activate Src. For instance, when the SH3 domain of Src interacts with the tail of the $\beta 3$ integrin, following integrin clustering, phosphorylation of Src at Tyr⁴¹⁶ is induced.

Still controversial is the mode of integrin-dependent Src activation and its localization at particular intracellular sites. One model holds that Pyk2 and c-Cbl mobilize c-Src to the integrin (Sanjay, et al., 2001). Upon adhesion, Pyk2 Tyr⁴⁰² becomes phosphorylated and activates c-Src by occupying its SH2 domain. However, this scenario does not explain why the bone phenotype of Cbl or Pyk2 deficient animals is not as profound as that attending Src deletion, or why Pyk2 phosphorylation is normal in $\beta 3$ integrin null OCs, in which Src and Cbl activation is downmodulated (Chiusaroli, et al., 2003; Gil-Henn, et al., 2007). An alternative hypothesis posits that c-Src is an upstream mediator of Pyk2 activation. Rodan's group demonstrated that following $\alpha \nu \beta 3$ integrin engagement, c-Src is phosphorylated at Tyr⁴¹⁶, activating its kinase activity and inducing an interaction between the SH2 domain of Src and Pyk2 (Lakkakorpi, et al., 2003). Calcium signals lead to Pyk2 autophosphorylation and subsequent further tyrosine phosphorylation by Src and mobilization to the integrin.

From all the above studies it appears that the mechanistic relationship between Src and Pyk2 is complex. The activity of each is diminished in the absence of the other, suggesting mutual dependence. It is surprising that despite the fact that Pyk2 is an integral component of the adhesive complex, the bone phenotype of Pyk2 deficient mice is not as dramatic as c-Src or $\alpha \nu \beta 3$ -/- mice.

Phospholipase C (PLC) $\gamma 2$ is an additional molecule shown to regulate Src activation and localization with $\alpha \nu \beta 3$ and Pyk2 (Epple, et al., 2008). PLC $\gamma 2$ can generate PIP2 and DAG, thereby increasing intracellular calcium, and also acts as an adapter protein via its SH2, SH3, and pleckstrin homology domains. Association between PLC $\gamma 2$ SH2 motifs and Pyk2 in OCs is dependent on adhesion (Nakamura, et al., 2001). Mice deficient for PLC $\gamma 2$ are osteopetrotic due to inhibition of OC differentiation (Mao, et al., 2006). By using PLC $\gamma 2$ -deficient OC precursors overexpressing NFATc1 to circumvent their blockade in OC differentiation, it has been shown that PLC $\gamma 2$ is critical for Src phosphorylation and its recruitment to the $\alpha \nu \beta 3$ adhesion complex. In PLC $\gamma 2$ -/- OCs, Src is randomly distributed on the cell membrane without localizing to the peripheral podosome belt. While not affecting Pyk2 localization, PLC $\gamma 2$ is still required for its phosphorylation and association with c-Src and $\alpha \nu \beta 3$. Consequently, NFATc1-rescued PLC $\gamma 2$ -deficient OCs do not migrate or spread normally or resorb bone (Epple, et al., 2008).

Both the catalytic activity and adapter functions of PLC $\gamma 2$ may be required for Pyk2 activation and consequently Src localization. Pyk2 autophosphorylation at Tyr⁴⁰² is a calcium-dependent event that occurs independent of Src activity (Sanjay, et al., 2001). PLC $\gamma 2$ -null cells have decreased levels of Pyk2 -phosphoTyr⁴⁰² in response to integrin engagement (Epple, et al., 2008), which may contribute to abnormal Src localization in these cells. Furthermore, treatment with the PLC γ inhibitor U73122, which likely influences Tyr⁴⁰² phosphorylation of Pyk2, disrupts the Src/Pyk2/ $\beta 3$ adhesive complex in adherent WT OCs. In further support of a role for the lipase activity of PLC $\gamma 2$, OCs carrying the PLC $\gamma 2$ catalytic domain mutant display improper Src localization. However, the adapter function of PLC $\gamma 2$ also appears to modulate the $\alpha \nu \beta 3$ adhesive complex. Mutation in the SH2, but not SH3, domains of PLC $\gamma 2$ abrogates Src activation, localization and consequently the formation of a sealing zone. Thus, while the catalytic activity of PLC $\gamma 2$ enhances Pyk2 phosphorylation via calcium signals, PLC $\gamma 2$, via its SH2 motif, can also directly bind to Pyk2, recruiting it and Src to the integrin complex.

The mechanism of PLC γ 2 regulation in response to adhesion remains to be fully elucidated. Considering that ITAM (immune tyrosine-based activating motif)-containing receptors, such as DAP12 and FcR γ , modulate PLC γ 2 phosphorylation in neutrophils, macrophages, platelets, and osteoclasts (Jakus, et al., 2007), it is likely that these receptors also control PLC γ 2 activation in response to integrin engagement (Figure 2).

DAP12: a point of convergence between α v β 3 and M-CSF signaling pathways

DAP12, a transmembrane adapter molecule expressed in immune cells, is one common orchestrator of growth factor and integrin signals (Lanier and Bakker, 2000; Tomasello, et al., 1998). In myeloid cells, DAP12 pairs with surface residing receptors including triggering receptor expressed on myeloid cells and osteoclasts (TREM2) (Colonna, 2003). The cytoplasmic domain of DAP12 contains the ITAM motif, which when phosphorylated functions as a docking site for tyrosine kinases, including Syk (McVicar, et al., 1998). Deletion of the DAP12 gene, in man, results in Nasu-Hakola disease, which includes skeletal abnormalities in its phenotype (Hakola, 1972). Furthermore, DAP12 is expressed by OCs and, as evidenced by the development of mild osteopetrosis in mice lacking the protein, is important for normal resorptive function (Kaifu, et al., 2003; Kondo, et al., 2002).

Due to the presence of another ITAM-containing receptor on the OC surface, FcR γ , DAP12-deficient precursors differentiate normally in vitro. However, they appear smaller, do not form podosome belts, and lack the ability to resorb bone (Faccio, et al., 2003b; Zou, et al., 2007; Zou, et al., 2008). The functional defect of DAP12 $^{-/-}$ OCs is due to impaired M-CSF and α v β 3 integrin signaling. In response to integrin engagement or M-CSF stimulation, the 2 tyrosine residues of the DAP12 ITAM motif are phosphorylated by c-Src. The modified ITAM can then bind another non-receptor tyrosine kinase, Syk and trigger a cascade of signals, including PLC γ 2 activation, which culminates in cytoskeletal reorganization. Syk is directly phosphorylated by c-Src following α v β 3 integrin engagement. Mutation in the Syk SH2 motifs which abrogates the capacity of the molecule to bind to DAP12 while retaining binding with α v β 3 disrupts the association of Src and Syk and thus the OC cytoskeleton (Zou, et al., 2008). The Syk $^{-/-}$ OC phenotype resembles that of β 3 integrin-deficient cells, as Syk $^{-/-}$ preosteoclasts show defective spreading and adhesion when plated on α v β 3 integrin-ligand surfaces and do not resorb bone (Faccio, et al., 2003b). Although lethal in utero, the in vivo bone phenotype of Syk $^{-/-}$ embryos or chimeric mice bearing Syk $^{-/-}$ hematopoietic cells also manifest defective bone resorption (Zou, et al., 2007). In addition to its activation by integrin adhesion, Syk also becomes activated by M-CSF stimulation in a DAP12-dependent manner, although in this circumstance the mechanism is autophosphorylation rather than trans-phosphorylation by Src (Zou, et al., 2008). Mutation in the SH2 motif of Syk abrogates its binding to the ITAM motif of DAP12 and dampens the response to M-CSF. Thus, the Syk/DAP12 association represents a point of convergence between M-CSF and α v β 3 signaling to the cytoskeleton.

Downstream mediators of motility/cytoskeleton in the OC

The key effector for cytoskeletal rearrangement in OCs immediately downstream of Syk, and therefore M-CSF, α v β 3 integrin and DAP12, is Vav3. Syk activates Vav3 by direct phosphorylation. Vav3 null mice have increased bone mass due to decreased bone resorption by OCs (Faccio, et al., 2005). In vitro, Vav3 $^{-/-}$ OCs differentiate normally, but they fail to form podosome belts on glass or a sealing zone on bone, and have little resorptive activity.

Vav3 is a guanine nucleotide exchange factor (GEF) for the Rho family small GTPase Rac. GTPases such as Rac are regulated by the type of guanine nucleotide bound; GTP-bound

Rac is active, while the GDP-bound form is inactive. GEFs such as Vav3 bind GDP-Rac, causing a conformational change that favors release of GDP and binding of GTP, which is present at high concentrations in the cell. Rac is also regulated by post-translational addition of a geranylgeranyl moiety at its C-terminus, which allows insertion into the plasma membrane. The intrinsic GTPase activity of Rac returns it to the GDP-bound inactive form, a process which is accelerated by binding of another class of regulators, the GTPase activating proteins (GAPs). However, a specific GAP for Rac that is active in OCs has not yet been described. In most cell types, Rac is important for cell motility because it regulates formation of lamellipodia at the leading edge, and promotes formation and turnover of adhesive complexes such as podosomes (Ridley, et al., 2003).

Rac has 3 isoforms, but only Rac1 and Rac2 are present in OCs and their precursors. Rac1 is expressed at higher levels than Rac2 in this lineage (Wells, et al., 2004; Yamauchi, et al., 2004). Studies of mice lacking Rac1 and Rac2, alone or in combination establish some compensation between the isoforms. Deletion of both is required to generate a significant increase in bone mass in vivo. Rac1/2 double-deficient OC precursors are smaller, and have fewer lamellipodia than wildtype controls. They also migrate poorly in response to M-CSF, with the latter effect being controlled only by Rac1 (Wang, et al., 2008). Adenovirus vector-mediated expression of dominant-negative Rac1 reduces membrane ruffling and spreading of OCs in response to M-CSF, suggesting that Rac controls OC motility as well as spreading (Fukuda, et al., 2005). Further supporting evidence of the role of Rac in OC motility comes from neurofibromatosis type 1 (Nf1). Nf1^{+/-} OCs have increased motility and podosome belts, resulting in increased resorptive activity. In mice, removing Rac1 from Nf1^{+/-} cells normalized actin organization and OC motility (Yan, et al., 2008).

Activated Rac stimulates many downstream effectors, several of which regulate the formation or stabilization of F-actin. However, Rac-activated pathways have not been defined in the OC.

cdc42

cdc42 is another Rho family small GTPase which, when overexpressed as an activated mutant form in macrophages and OCs disrupts podosomes, depleting the core of F-actin (Chellaiah, 2005; Linder, et al., 1999). However, more recent studies using mice with increased cdc42 activation due to knockout of its negative regulator cdc42GAP have shown increased sealing zone formation and bone resorption, compared to wildtype cells. Additionally, the formation of the Par3/Par6/aPKC polarity complex downstream of RANKL in OCs seems to depend on cdc42 (Ito, et al., 2007), further supporting the important role of cdc42 in OC activity.

Cdc42 binds directly to Wiscott Aldrich Syndrome protein (WASP) (Chellaiah, 2005; Rohatgi, et al., 1999), a multi-domain adapter protein regulating transmission of signals to the actin cytoskeleton. WASP depletion in macrophages leads to a virtual absence of podosomes (Jones, et al., 2002) and a defective chemotactic response under a gradient of M-CSF. Similarly, WASP deficient OCs are depleted of podosomes and fail to assemble normal actin rings on bone, resulting in impaired resorption (Calle, et al., 2004). Although physiological steady-state levels of bone resorption are maintained, a major impairment is observed when WASP-null animals are exposed to a resorptive challenge (Calle, et al., 2004). Interestingly, when PIP2 is bound to WASP, its interaction with cdc42 is enhanced. Furthermore, PIP2 levels are affected by the activity of RhoA (Chellaiah, 2005).

One important function of WASP is to activate the actin-related protein (Arp) 2/3 complex, required for de novo actin polymerization. The Arp2/3 complex is essential for formation of actin trimers, which can quickly be assembled into actin filaments, generating the force

necessary for membrane protrusions. Small interfering RNA suppression of the Arp2/3 complex (Hurst, et al., 2004) disrupted podosomes and actin ring formation in OCs.

Another protein important for actin dynamics in the OC is cortactin (Tehrani, et al., 2006), which directly binds both Arp2/3 and F-actin. Cortactin, whose expression is upregulated during OC differentiation, is a prominent Src substrate (Maa, et al., 1992). Depletion of cortactin from OCs with shRNA results in a loss of podosomes and membrane protrusions, resulting in a lack of a sealing zone and ablation of bone resorption (Tehrani, et al., 2006). A cortactin mutant that could not be phosphorylated by Src localizes normally but does not rescue podosome formation in cortactin-depleted OCs.

RANKL and the OC cytoskeleton

In addition to its critical role in OC differentiation, RANKL has also been linked to control of the cytoskeleton. Addition of RANKL to mature OCs on bone enhances actin ring formation and bone resorption (Burgess, et al., 1999). With RANKL stimulation of OCs expressing RANK mutants that are unable to interact with TRAF6, F-actin is found in clumps rather than rings, and c-src and c-cbl are mislocalized. As expected, bone resorption is impaired (Armstrong, et al., 2002). Recently, a cell-permeable peptide corresponding to a non-TRAF binding RANK cytoplasmic motif was found to disrupt the OC cytoskeleton by blocking phosphorylation of Vav3, and preventing Rac and Cdc42 activation (Kim, et al., 2009). Addition of this peptide to mature OCs caused cell retraction, loss of the actin ring, and a dramatic reduction in bone resorption. This peptide did not affect Vav3 and Rac signaling initiated by M-CSF, indicating that these two cytokines can activate the same downstream pathways independently. Considering that RANKL-mediated osteoclastogenesis requires costimulation of ITAM receptors, DAP12 and FcR γ , it is very likely that these cooperative signals also modulate RANKL-dependent cytoskeletal changes, in a similar manner to that described for M-CSF signaling.

Conclusion

The most common bone disease affecting the aging population is osteoporosis, a disease in which bone resorption exceeds formation. Because the OC is responsible for bone resorption, it has been the focus of anti-osteoporosis therapies, and the most commonly used class of drugs is the nitrogen-containing bisphosphonates. These drugs target FPP synthase, an enzyme in the cholesterol biosynthesis pathway required for the post-translational modification of small GTPases (including Rho, Rac, and cdc42) which allows their membrane insertion. Because appropriate localization at the membrane is critical for these small GTPases, which themselves are required for regulation of the OC cytoskeleton, bisphosphonates disrupt the OC cytoskeleton and reduce resorptive activity. Thus, the machinery of OC motility is an effective therapeutic target, and a better understanding of this complex process may contribute to even more effective treatments. One area that is still understudied is the motion of OC on bone, especially *in vivo*. Hopefully, advances in *in vivo* microscopy will provide insights into the activity of the OC in its natural environment.

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Figure 1.

The sealing zone in resorbing OCs. **Left**, An OC on bone is a tall polarized cell with its robust actin ring (green) forming the backbone of the sealing zone, and area of membrane tightly juxtaposed to the bone surface that defines the resorption lacuna (asterisk). Within the sealing zone is the specialized membrane organelle known as the ruffled border (arrowhead), formed by fusion of intracellular vesicles containing the enzymes and transmembrane proteins needed for bone resorption. A FITC-phalloidin stained OC plated on bone, demonstrating a single actin ring, is shown below. **Right**, As it resorbs, an OC moves, with forward membrane extensions (solid arrow). New sealing zones form, encircling a ruffled border, and forming a trail of resorption lacunae. Old sealing zones (dotted arrow) toward the rear of the cell dissolve. Each OC can have several actin rings, as shown in the FITC-phalloidin stain of OCs on bone, below.

Figure 2.

Signaling complexes assembled at c-Fms and $\alpha v \beta 3$, and downstream effectors to the cytoskeleton in the OC. Right, The cytoplasmic tail of c-Fms (the receptor for M-CSF) recruits a complex of Src, Cbl, and PI3K anchored at Tyr(Y)559, and stabilized by Y697 and Y721. This complex interacts with the ITAM-containing adapter DAP12, which associates with Syk to initiate downstream signals to the cytoskeleton. Left, The cytoplasmic tail of $\beta 3$ integrin, binds a complex containing Src, Cbl, Pyk2, and PLC γ 2, which also interacts with DAP12-associated Syk to activate cytoskeleton-modifying pathways. Vav3, an upstream regulator of Rac1/2, is activated by both pathways and is a direct target of Syk. The $\alpha v \beta 3$ integrin and c-Fms complexes can also activate cdc42 and WASP, as well as cortactin, which along with Rac lead to Arp2/3 dependent changes in F-actin.

Figure 3.

Cytoskeletal changes in migrating OCs on glass.

Left, OCs on glass or plastic are flat, nonpolarized cells. Their peculiar characteristic is the presence of a peripheral ring of actin formed by aligned podosomes, short lived and highly dynamic actin structures. Each podosome is composed of a bundle of perpendicular actin filaments (green dots) surrounded by $\alpha v \beta 3$ integrin (red circle) and actin binding proteins. Microtubules (black lines) associate with, and are thought to stabilize, the actin ring. **Right,** Extracellular stimuli, including M-CSF, can lead to rapid actin reorganization. The peripheral ring of actin dissolves, podosomes redistribute throughout the cell and polarized membrane extensions called lamellipodia form. F-actin (green line) is present at the leading edge of the lamellipodia, where it is associated with activated $\alpha v \beta 3$ integrin (red line).