CD44 and $\beta 3$ Integrin Organize Two Functionally Distinct Actin-based Domains in Osteoclasts

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The actin cytoskeleton of mature osteoclasts (OCs) adhering to nonmineralized substrates is organized in a belt of podosomes reminiscent of the sealing zone (SZ) found in bone resorbing OCs. In this study, we demonstrate that the belt is composed of two functionally different actin-based domains: podosome cores linked with CD44, which are involved in cell adhesion, and a diffuse cloud associated with $\beta 3$ integrin, which is involved in cell adhesion and contraction. Wiskott Aldrich Syndrome Protein (WASp) Interacting Protein (WIP)–/– OCs were devoid of podosomes, but they still exhibited actin clouds. Indeed, WIP–/– OCs show diminished expression of WASp, which is required for podosome formation. CD44 is a novel marker of OC podosome cores and the first nonintegrin receptor detected in these structures. The importance of CD44 is revealed by showing that its clustering restores podosome cores and WASp expression in WIP–/– OCs. However, although CD44 signals are sufficient to form a SZ, the presence of WIP is indispensable for the formation of a fully functional SZ.

INTRODUCTION

Podosomes are highly dynamic adhesion structures found in monocyte-derived cells and in v-src-transformed fibroblasts, and they can be induced by cytokines or phorbol esters in vascular cells. They are thought to carry out two major functions: adhesion and extracellular matrix (ECM) degradation (Tarone *et al.*, 1985; Correia *et al.*, 1999; Linder *et al.*, 1999, 2007a; Burns *et al.*, 2001; Moreau *et al.*, 2003; Varon *et al.*, 2006). Recently, podosomes found in leukocytes have been shown to initiate transcellular diapedesis (Carman *et al.*, 2007). Podosomes are formed by an F-actin core surrounded by several focal adhesion proteins such as integrins, vinculin, paxillin, and talin (Marchisio *et al.*, 1988; Zamboni-Zallone *et al.*, 1989; Pfaff and Jurdic, 2001; Linder and Aepfelbacher, 2003; Linder, 2007a). However, podosomes clearly differ from focal adhesions by their shorter lifetime and by the architectural organization of these proteins. Actin-associated proteins such as cortactin, Arp2/3,

and Wiskott Aldrich Syndrome Protein (WASp) are associated with the F-actin core. WASp is associated with WASp Interacting Protein (WIP), which exerts its functions by regulating the spatial organization of actin polymerization in all cell types studied so far (Anton and Jones, 2006). WIP can also interact with other proteins implicated in podosome formation such as cortactin or actin itself (Ramesh *et al.*, 1997; Martinez-Quiles *et al.*, 2001; Kinley *et al.*, 2003; de la Fuente *et al.*, 2007). WIP has indeed been detected in the podosomes of endothelial cells (Moreau *et al.*, 2003) and more recently in dendritic cells (Chou *et al.*, 2006) where it was found to regulate podosome formation. In these cells, knock down of WIP resulted in a decreased number of podosomes (Chou *et al.*, 2006). However, the role of WIP in osteoclasts (OCs) has not yet been investigated.

OCs are giant multinucleated bone-resorbing cells derived from the fusion of monocytic precursors stimulated by the receptor activator of nuclear factor B-ligand (RANK-L) and macrophage colony-stimulating factor (M-CSF) (Lacey *et al.*, 1998; Boyle *et al.*, 2003). OCs can adhere to several substrates on which they form distinct F-actin structures. During the *in vitro* differentiation of OCs, cells exhibit numerous podosomes that self-organize along the differentiation process. Podosome clusters are found in early OCs, which evolve into dynamic and transient rings at intermediate stages and

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end up forming peripheral podosome belts in mature cells (Destaing *et al.*, 2003). Based on in vitro observations, it has been proposed that podosomes are essential for ECM degradation, invasion, and migration in vivo (Mizutani *et al.*, 2002; Calle *et al.*, 2006; Redondo-Munoz *et al.*, 2006; Tatin *et al.*, 2006; Linder, 2007b). In vivo, bone resorbing OCs exhibit a sealing zone (SZ), made of a large circular band of F-actin with an inner and outer lining of vinculin (Lakkakorpi *et al.*, 1991; Saltel *et al.*, 2004; Jurdic *et al.*, 2006; Luxenburg *et al.*, 2007). The SZ delineates the area of enzyme secretion and the transcytosis of degraded materials occurs from basal-to-apical membranes (Nesbitt and Horton, 1997; Salo *et al.*, 1997). OCs adhering to glass spread and form podosome belts, whereas they become polarized and exhibit SZ when seeded onto apatite (Saltel *et al.*, 2004). The spatiotemporal relationship between these two structures formed in cultured and bone attached osteoclasts is still open to conjecture. Whereas no individual podosomes could be detected at the SZ formed in OCs seeded on a mineralized substrate, by means of time-lapse video confocal microscopy (Saltel *et al.*, 2004), a novel approach based on high-resolution electron microscopy revealed that the structure is made of a highly dense network of podosomes interconnected with radial bundles of actin (Luxenburg *et al.*, 2007).

OC adhesion is critical for its function of bone resorption and OCs express several matrix receptors, including $\alpha v \beta 1$, $\alpha 2 \beta 1$, and $\alpha v \beta 3$ integrins (Clover *et al.*, 1992; Nesbitt *et al.*, 1993). Although $\alpha v \beta 3$ integrins were described to be the most implicated in OC biology (Faccio *et al.*, 2003a,b; Zou *et al.*, 2007), another major receptor is represented by CD44 (Chellaiah *et al.*, 2003a,b). CD44 is a cell surface, single-pass transmembrane proteoglycan expressed in most cell types. The roles played by CD44 are diverse, but great interest in CD44 results from its function as a receptor for hyaluronic acid (HA) (Ponta *et al.*, 2003). Although HA is the principal ligand for CD44, it also interacts with osteopontin (OPN), collagen, and laminin (Goodison *et al.*, 1999).

In the present study, we analyzed the molecular composition and F-actin organization of the OC podosome belts. We first confirmed that the belt is composed of two distinct entities, the podosome core visualized as an F-actin dot that we described herein associated with CD44 and an actin cloud surrounding the core and that matched the $\beta 3$ integrin pattern. This finding demonstrates for the first time the presence of CD44 in podosomes, and it describes the first nonintegrin receptor in the podosome core. Interestingly, podosome core formation, but not the actin cloud, was abrogated in OCs obtained from WIP^{-/-} mice, demonstrating a structural independence between these different F-actin structures. The absence of podosomes could be explained by decreased WASp expression levels in WIP^{-/-} OCs. In addition, by providing CD44 signals, we were able to reinduce podosomes in WIP^{-/-} OCs, and the reappearance of podosomes was correlated with WASp recovery, indicating that CD44 activation could regulate WASp and promote podosome formation in the absence of WIP. We further show that podosome cores and actin clouds are involved in distinct functions in OC physiology where podosome cores play a major role in adhesion whereas the actin cloud controls contractility. We conclude that the clustering and activation of CD44 is sufficient to form a SZ. However, because WIP^{-/-} OCs displayed reduced resorbing activities, we demonstrate that the presence of WIP is necessary for the formation of a fully functional SZ.

MATERIALS AND METHODS

Mice

The generation of WIP^{-/-} mice has been described previously (Anton *et al.*, 2002). Mice are from a mixed 129/SvJ $\times$ C57Bl/6 background. They were kept under specific pathogen-free conditions at the Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain). Comparisons are made between cells obtained from age and sex-matched WIP^{-/-} and wild-type (WT) mice.

Cell Cultures

Spleen and bone marrow cells of WT and WIP^{-/-} mice were cultured for 8 d in six-well or 12-well plates in differentiation medium: α -minimal essential medium (α MEM) containing 10% fetal bovine serum, M-CSF, and RANK-L (Destaing *et al.*, 2003). For Western blot analysis, OCs were rapidly washed in ice-cold phosphate-buffered saline (PBS), and proteins were extracted with lysis buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1% Triton X-100, 10% glycerol, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, and 1 μ g/ml each of leupeptin, pepstatin, and aprotinin).

Reagents

Recombinant human RANK-L and human M-CSF were produced as described previously (Destaing *et al.*, 2003). Culture media were from Invitrogen (Invitrogen). Anti-WIP antiserum was raised by Proteintools (Madrid, Spain) by immunizing rabbits with a 19-amino acid C-terminal peptide of WIP (KLARNESRSGSNRRERGGP) (Lanzardo IJBCB 39 2007). Anti-paxillin (clone 349) was from BD Transduction Laboratories (Lexington, KY). Anti-phosphopaxillin was from Invitrogen (Carlsbad, CA). Anti-vinculin (clone Vin 11-5), anti- α -actinin (clone BM-75.2), and anti-myosin (clone MY-32) were from Sigma-Aldrich (St. Louis, MO). Anti-CD44 (H-CAM [H-300], sc-7946; H-CAM [F-4] sc-9960) and anti-WASp (sc-13139) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Cortactin (p80/85; clone 4F11) and anti-phosphotyrosin (clone 4G10) were from Upstate Biotechnology (Charlottesville, VA). Anti-CD61 (integrin $\beta 3$ subunit) and anti-CD29 (integrin $\beta 1$ chain; clone 9EG7 and clone Ha2/5) were from BD Biosciences Pharmingen (San Diego, CA). Anti-Arp3 was a generous gift from E. Znameroski (Welch laboratory, University of California, Berkeley, CA). Alexa Fluor-555-Phalloidin was from Molecular Probes (Eugene, OR). The myosin inhibitors blebbistatin (Sigma-Aldrich) and ML-7 (Calbiochem, San Diego, CA) were used for 20 min at 50 and 0.5 μ M respectively. Vitronectin (VN), collagen, and laminin were obtained from Sigma-Aldrich, and they were incubated on glass slides at 10 μ g/ml for 1 h. Osteopontin and hyaluronic acid (Sigma-Aldrich) were incubated on glass slides at 5 μ g/ml for 1 h. After rinsing, coated slides were blocked for 1 h in bovine serum albumin 0.5% in PBS (Sigma-Aldrich). To study the blocking of CD44 or $\beta 3$ integrin subunit signaling, OCs were incubated with 10 μ g/ml CD44 blocking antibodies for 20 min (sc-7946; Santa Cruz Biotechnology), or 10 μ M cyclic Arg-Gly-Asp (cRGD) peptides (Bachem, Bubendorf, Switzerland; Legler *et al.*, 2001) for 30 min, respectively. For the activation of CD44 signaling, OCs were incubated on glass coverslips coated first with 10 μ g/ml poly-L-lysine (Sigma-Aldrich) for 2 h and then overnight with a CD44-activating antibody (clone A3D8; Sigma C7923). Cell cultures were fixed 4 h after being seeded on these matrices.

Immunofluorescence

Cells were fixed in 3.7% paraformaldehyde, pH 7.2, for 10 min, permeabilized with 0.2% Triton X-100 for 7 min, and incubated with various antibodies. F-actin distribution was revealed by Alexa Fluor-555-Phalloidin. Cells were imaged with a confocal LSM 510 (Carl Zeiss Microimaging, Jena, Germany) by using a 63 $\times$ (numerical aperture [NA] 1.4) Plan Neofluar objective. To prevent contamination between fluorochromes, each channel was imaged sequentially using the multitrack recording module before merging. Z-cut pictures were obtained using LSM 510 software. Deconvoluted images were obtained from Z-cut pictures (X = 40 nm, Y = 40 nm, Z = 131 nm), by using Huygens essential software (BioVision Technologies, Exton, PA).

Interference Reflection Microscopy (IRM)

The contact areas of WT or WIP^{-/-} OCs were assessed by IRM. Adherent cells were imaged with an Antiflex Plan-Neofluor Ph3, 63 $\times$ NA 1.25 oil immersion objective, equipped with a $\lambda/4$ plate and custom-made polarization and analysis filter, mounted on an Axiovert 100M (both from Carl Zeiss Microimaging). Images were captured with a 12-bit digital charge-coupled device camera (model ORCA 4742-95; Hamamatsu Photonics, Massy, France), controlled by Openlab software (Improvision, Coventry, United Kingdom). Image analysis was performed using the MetaMorph software package (Molecular Devices, Sunnyvale, CA).

Measurement of OC Surface Areas

OCs were stained for F-actin and imaged with a 20 $\times$ (NA 1.0) Plan-Achromat objective (Carl Zeiss Microimaging). OC surface area was measured using MetaMorph software. Results are plotted as the mean number of pixels included in the OC surface area, $\pm$ SD, out of three wells.

Measurement of Resorption Efficiency

At the end of the differentiation process on plastic dishes, mature OCs were removed with four washes with PBS– 0.25 mM EDTA for 30 min each and then seeded on various substrates. OCs were seeded on dentin slices (a generous gift from Dr N. Takahashi, Matsumoto Dental University, Nagano, Japan) or on apatite collagen complex (ACC) slides in differentiation medium for 2 d. ACC was prepared using the method described previously (Shibutani *et al.*, 2000; Saltel *et al.*, 2004). Remaining cells were removed by immersion in water, and ACC slides were stained with silver nitrate to detect resorption pits under a light microscope. Resorbed areas were assessed using a stereomicroscope (MZ12; Leica, Wetzlar, Germany) and using MetaMorph and Photoshop software (Adobe Systems, Mountain View, CA). Results were plotted as the mean number of pixels $\pm$ SD out of three wells, and they were representative of three independent experiments.

Measurement of F-Actin Intensities

WT and WIP–/– cells were fixed and stained for F-actin. Thirty pictures of the actin cytoskeleton were acquired at high magnification with identical excitation and exposure settings by using the confocal microscope described above. The intensity of every single pixel was measured with MetaMorph software, as described by Cluzel *et al.* (2005). After smoothing (3×3 kernel), background subtraction, and manual selection of the cell surface using MetaMorph software, data were exported to Excel (Microsoft, Redmond, WA) for further analysis. Cumulative fluorescence intensity histograms were obtained by multiplying the number of pixels with their respective gray value. This measurement was repeated on 30 different podosome belts, and an average graph was drawn. The intensity limit between actin cloud and podosome cores was fixed at an intensity value of 150, corresponding to the separation between the curve and its tangent.

OC Microinjections

Mouse spleen cell-derived OCs, differentiated in vitro on Eppendorf CELLocate coverslips for 7 d in differentiation medium, were transferred to medium buffered with 20 mM HEPES, pH 7.4. Intranuclear microinjections of cDNA (3.5 mg/ml in 0.05 M Tris-HCl, pH 7.4) were carried out at room temperature using an Eclipse TE 200 inverted microscope (Nikon, Tokyo, Japan) with an InjectMan micromanipulator and an Eppendorf 5246 microinjector. After injection, cells were further maintained at 37°C and 5% CO₂ for 6 h in differentiation medium, before imaging.

OCs were transfected with either control plasmid (pcDNA), or with plasmid containing the human WIP coding sequence (pcDNA-WIP), as described previously (Anton *et al.*, 2002). These plasmids were kindly provided by N. Ramesh (Children's Hospital, Boston, MA).

RESULTS

CD44 and $\beta 3$ Integrin Receptors: Mediators of Two Distinct F-Actin–based Domains of the Podosome Belt

Podosomes are considered as the structural unit of the OC actin cytoskeleton in vitro. During osteoclastogenesis, the pattern of podosome organization evolves from clusters in the early stages to expanding dynamic rings at intermediate stages and finally to belts localized at the cell periphery in mature OCs (Destaing *et al.*, 2003). To get more insight into the organization of F-actin in this continuously evolving structure, phalloidin labeling was performed and analyzed using confocal microscopy. Data presented in Figure 1 revealed that the podosome belt (Figure 1A) was, in fact, composed of two distinct F-actin entities (Figure 1B). Strong fluorescence intensity matching dense dot-like fibrillar actin (Figure 1B, black star), delimited the podosome cores, which were found connected with a more diffuse, low-intensity F-actin staining (Figure 1B, white star), which we defined as the actin cloud. To examine the contribution of these two distinct structures to the podosome belt, the pixel/intensity distribution from >30 cells was quantified, averaged, and expressed in the form of a histogram, reflecting the surface and relative fluorescence intensity of these two domains (Figure 1C). A Z-section of a podosome belt showed podosome cores as intense columns of fibrillar actin, whereas the actin cloud manifested itself as a thin continuous network connecting podosomes with each other (Figure 1D, top). Deconvolution analysis of this Z-section confirmed that podosomes and clouds were interconnected (Figure 1D, bottom). These data unambiguously established that podosome belts of

mature OCs, presented two distinct structural arrangements of F-actin consisting of the podosome core embedded in a cloud of polymerized actin. Because OC adhesion plays a crucial role in the bone resorption process, we next examined the localization of receptors for components of the extracellular bone matrix expressed in this cell type, namely, $\alpha v\beta 1$, $\alpha 2\beta 1$, and $\alpha v\beta 3$ integrins and CD44 (Clover *et al.*, 1992; Nesbitt *et al.*, 1993; Chellaiah *et al.*, 2003b). We were unable to detect $\beta 1$ subunits with two antibodies (9G7 and Ha2/5), which easily reacted with murine fibroblasts (data not shown). $\alpha v\beta 3$ integrins and CD44 receptors showed specific and nonoverlapping distribution profiles within the cells. Interestingly, CD44 selectively localized to podosome cores (Figure 1E). This finding identifies CD44 as the first nonintegrin receptor at the podosome core. In contrast, $\beta 3$ subunit staining matched perfectly that of the actin cloud, excluding actin dots corresponding to podosome cores (Figure 1F). Because actin dots and clouds colocalized with distinct receptors, we hypothesized that the formation of the two structures may emanate from the activation of distinct signaling pathways.

WIP Null OCs Are Devoid of Podosome Cores

The Arp2/3/WASp/WIP machinery regulates actin polymerization at podosomes (Linder *et al.*, 2000, 2007a; Tsuboi, 2007). Because dendritic cells from WIP knockout mice were recently reported to display decreased podosome numbers (Chou *et al.*, 2006), we designed experiments to analyze the impact of the loss of WIP on the two F-actin structures. Mature OCs, derived from splenic cells of WIP–/– animals were grown in the presence of M-CSF and RANK-L and then seeded onto serum-coated glass coverslips. Phalloidin staining revealed that WIP–/– OCs conserve a belt of F-actin (Figure 2A). However, observation of the belt at higher magnification showed an absence of actin dots (Figure 2B). Furthermore, histogram analysis revealed the structural conservation of the actin cloud, whereas podosome cores were lost (Figure 2C). Considering the integrated fluorescence intensity as a measure of the quantity of F-actin, the overall amount of fibrillar actin seemed very similar between WT and WIP–/– OCs (664×10^4 pixels for the WT vs. 628×10^4 pixels for WIP–/– OCs). The analysis of a Z-section confirmed the absence of podosomes and the homogeneity of the observed F-actin structures (Figure 2D, top). Image deconvolution (Figure 2D, bottom) confirmed that this structure consists of a large band of fibrillar actin and revealed areas of higher intensity corresponding to radial bundles of actin. To confirm the role of WIP in podosome formation in our model, WIP–/– OCs were microinjected with a construct encoding a full-length WIP-green fluorescent protein (GFP) cDNA, and they were stained for F-actin 6 h later. As expected, reinduction of podosomes was observed in WIP-GFP-positive cells (Figure 2, E and F), confirming that the absence of podosome cores is due to WIP deficiency. These data show the specific role of WIP in podosome formation in OCs, in accordance with results established in dendritic cells.

To explore more specifically the role played by WIP in OCs, the contribution of WIP to the discrete structures forming the podosome belt was analyzed. The expression and localization of CD44 and $\beta 3$ integrin, used as selective markers of each structure, were investigated by Western blot and immunofluorescence, respectively. The absence of WIP (Figure 2G) and the expression of CD44 and $\beta 3$ integrins in WIP–/– OCs (Figure 2, H and I) were first confirmed by Western blot. However, the immunofluorescence approach revealed that $\beta 3$ staining remained colocalized with the actin cloud at the cell periphery (Figure 2I), but CD44 stain-

Figure 1. OC podosome belt is composed of two F-actin domains specifically associated with CD44 and the $\beta 3$ integrin subunit. (A) Mature WT OCs plated on glass coverslips were observed by confocal microscopy after F-actin staining with phalloidin, and they exhibited podosome belts. Bar, 20 μm . (B) Magnified deconvoluted view of the area within the white inset in A shows that podosome belts are formed by two F-actin-containing structures: the cloud (white star) and dots (black star), referred to as podosome cores, embedded in the cloud. Bar, 1.5 μm . (C) Cumulative fluorescence intensity histograms were obtained by multiplying the number of pixels by the respective gray value of each pixel obtained from 30 images. This analysis illustrates the difference between podosome clouds and podosome cores ($n = 30$). (D) Z-cut section of a magnified view of the F-actin cytoskeleton (dotted white line in B) before (top) and after (bottom) deconvolution, confirmed the existence of the actin cloud, which interconnects with the actin dots of podosome core fluorescence. Bar, 0.5 μm . (E and F) Differentiated wild-type OCs were observed after actin staining by phalloidin and immunolabeling for CD44 and the $\beta 3$ integrin subunit. CD44 receptor (E) colocalized with actin dots, whereas the $\beta 3$ integrin subunit (F) was specific to the actin cloud.

ing was diffusely distributed at the cell membrane (Figure 2H). The major alterations in the organization of the podosome belt resulting from the loss of WIP suggest that WIP is necessary to recruit and organize molecules during podosome formation.

CD44 Activation Restores Podosome Assembly in WIP $^{-/-}$ OCs

The localization of CD44 at the base of podosome cores in WT OCs suggested to us that this receptor may play a role in their formation. To analyze the contribution of CD44 in this process WT or WIP $^{-/-}$ mature OCs were seeded on various matrices displaying distinct CD44 binding capacities.

WT OCs exhibited podosomes on glass (Figure 3A) and on all other matrices tested (data not shown). In contrast, WIP $^{-/-}$ OCs were unable to assemble podosome cores on uncoated glass or VN, an $\alpha\text{v}\beta 3$ integrin ligand (Figure 3, B and C). However, despite the absence of WIP, podosome core assembly was restored when WIP $^{-/-}$ OCs were seeded on collagen I, OPN, or HA (Figure 3, D–F), all ligands for CD44. Consistent with this, CD44 colocalized with podosome cores in WIP $^{-/-}$ OCs seeded on HA (Figure 3F, inset). Furthermore, when plated on coverslips coated with an activating anti-CD44 antibody (clone A3D8), podosome formation was equally restored in WIP deficient OCs (Figure 3G). These results show that triggering CD44 signaling re-

Figure 2. WIP is required for podosome core formation. (A) Differentiated WIP^{-/-} OCs plated on glass coverslips were observed by confocal microscopy after F-actin staining by phalloidin and exhibited a continuous actin belt. Bar, 20 μ m. (B) Magnified deconvoluted view of the area within the white inset in A showed that the actin belt of the WIP^{-/-} OCs consisted of the cloud domain only (white star), with the complete absence of podosome cores. Bar, 3 μ m. (C) Comparison between cumulative intensity histograms (n = 30) of WT versus WIP

induced podosome cores in WIP^{-/-} OCs and confirm the direct implication of CD44 in podosome core formation in OCs. To investigate the mechanisms by which CD44 restores podosome core formation, WIP^{-/-} OCs were seeded either on coverslips where WT OCs had been cultured for 2 d, or with WT OC conditioned medium. Podosome cores were induced only in the first situation (Figures 3, H and I). This indicates that OCs provided CD44 activating signals, possibly by secreting insoluble matrix components necessary for CD44-mediated podosome core formation. In a converse experiment, WT OCs were treated with CD44 antibodies endowed with blocking properties (sc-7946). As expected, a dramatic decrease in podosome core numbers was observed (Figure 3, J and K) and confirmed by actin fluorescence quantitation (Figure 3L). Together, these data provide evidence that CD44 clustering promotes podosome core formation in WIP^{-/-} OCs, thereby bypassing the need for WIP during podosome formation.

CD44 Activation Compensates WIP Deletion by WASp Stabilization

Arp2/3/WASp/WIP and cortactin regulate actin polymerization at podosomes (Linder *et al.*, 2000; Tehrani *et al.*, 2006;

Webb *et al.*, 2006; Linder, 2007a; Tsuboi, 2007) and WASp plays a central role in podosome core formation (Linder *et al.*, 1999). Several studies have recently shown that WASp interaction with WIP protects WASp from degradation and subsequent podosome loss (Tsuboi, 2007) (Chou *et al.*, 2006). Our results show that, even in the absence of WIP, podosome cores form as long as CD44 signals are provided. To investigate WASp status in WIP^{-/-} OCs, the expression and localization of WASp and the other molecules implicated in podosome core formation were examined in WT versus WIP^{-/-} OCs. First Arp2/3, which was exclusively localized at podosome dots in WT OCs (Figure 4A), was not found in the actin cloud of WIP^{-/-} OCs (Figure 4B). Arp3 was still expressed but diffusely distributed at the cell membrane (Figure 4, B and C). Cortactin, which presents a similar pattern to Arp3 in WT OCs (Figure 4D), was also maintained, but it was confined to the actin cloud in WIP^{-/-} OCs (Figure 4E). Cortactin expression did not differ between WT OCs and WIP^{-/-} OCs (Figure 4F). WASp, which was localized at podosome cores in WT OCs (Figure 4G), was absent in WIP^{-/-} OCs (Figure 4H), as reported for WIP^{-/-} dendritic cells (Chou *et al.*, 2006). This result was confirmed by Western blot analysis, where WASp was un-

detectable in the WIP^{-/-} OC population (Figure 4I). To examine WASp status in conditions where WIP^{-/-} OCs assemble podosomes, WIP^{-/-} OCs were seeded on HA and labeled for WASp and Arp3. HA-induced podosomes were found to be WASp, Arp 3, and cortactin positive and their localization exactly matched podosome core staining (Figure 4, J and K; data not shown). Moreover, Western blot analysis performed on WIP^{-/-} OCs seeded on HA versus uncoated glass confirmed WASp expression when CD44 was clustered and activated (Figure 4L). All these data assess the importance of WASp/WIP interaction for podosome core formation in OCs. Interestingly, in this model, activated CD44 substitutes for WIP, to maintain WASp level and podosomes in these cells. These data establish that CD44/WASp/WIP are all involved in podosome core formation in OCs.

Podosome Cores and Actin Clouds Perform Distinct Functions in OCs

Podosome cores and actin clouds harbor specific receptors, CD44 and $\beta 3$ integrin, respectively, associated with specific molecular components, suggesting distinct roles for these two F-actin structures. Podosome cores are considered as adhesion structures in OCs, whereas the function of the actin cloud is unknown. WIP^{-/-} OCs offer the possibility to explore the function of each F-actin-based structure individually. To examine the role of podosome cores in cell adhesion, we first observed cells by IRM microscopy (Figure 5, A–D). In both cell types, IRM images revealed the presence of a large dark band at the cell periphery corresponding to tight attachment to the substratum, which colocalizes with the actin belt, with or without podosome cores (Figure 5, A–D). To gain information on the adhesion potential associated with these structures, a functional assay was set up. WT or WIP^{-/-} OCs were treated with EDTA (a specific method used to detach OCs) for either 7 or 15 min, and the remaining adherent OCs were recorded. WIP^{-/-} OCs, devoid of podosome cores, were found to be far less adhesive than WT OCs with podosome cores (Figure 5E). These data indicate that the actin cloud alone confers some adhesive function to the substratum and that podosome cores reinforce this potential. However, this result could be due to a specific effect of EDTA, a chelator of divalent cations known to be necessary for integrin function. To address more specifically the role of each receptor in adhesion, we used a blocking antibody targeting CD44 (Sc-7946), or peptide cRGD, a $\beta 3$ integrin inhibitor. Results presented in Figure 5F show that blocking $\beta 3$ integrin not only reduced the adhe-

Figure 2 (cont). ^{-/-} OC actin cytoskeleton confirmed the absence of the podosome core fluorescence domain, and the exaggerated fluorescence of the cloud domain. (D) Z-cut sections of a magnified view of the F-actin cytoskeleton before (top) and after (bottom) deconvolution confirmed that the actin cloud is homogeneous and devoid of any actin dots. Bar, 2 μ m. (E) Immature WIP^{-/-} OCs were microinjected with a WIP full-length cDNA and observed after F-actin and WIP staining: actin dots were reintroduced in microinjected cells. Bar, 5 μ m. (F) Magnified view of the area within the white inset in (E), showing podosome cores. Bar, 1.5 μ m. (G–I) Differentiated WIP^{-/-} OCs were observed after actin staining by phalloidin and immunolabeling for WIP, CD44, and $\beta 3$ integrin subunits. The expression level of these proteins was also revealed by Western blots. As expected, WIP (G), which colocalized with actin dots in WT OCs, was absent. CD44 receptor (H) was diffusely expressed at the cell membrane, whereas the $\beta 3$ integrin subunit (I) remained colocalized with the actin cloud.

Figure 3. CD44 plays a key role in the induction of actin dots. Mature WT OCs formed actin dots when seeded on different substrates such as glass (A) but also, vitronectin, collagen type I, OPN, and HA (data not shown). Mature WIP^{-/-} OCs formed an homogenous actin cloud but no podosome cores on glass (B) or vitronectin (C). In contrast, podosome cores were reintroduced when WIP^{-/-} OCs were seeded on different ligands of CD44: collagen type I (D), OPN (E), or HA (F). Bar, 4 μ m. The CD44 receptor colocalized with these podosome cores (inset). Bar, 8 μ m. Podosome cores were also reintroduced when mature WIP^{-/-} OCs were plated on coverslips, either coated with CD44-activating antibodies (A3D8) (G) or coated for 3 d with mature WT OCs, which were then removed (H); mature WIP^{-/-} OCs seeded on glass first coated with mature OC-derived conditioned medium, did not form podosome cores (I). Bar, 16 μ m. Insets show a magnified view of the actin belts under each condition. Bar, 8 μ m. (J) In contrast, incubation of WT OCs plated on glass with CD44 blocking antibodies (sc-7946) induced actin dot dissolution. Bar, 16 μ m. (K) Magnified view of the area within the white inset in J. Bar, 4 μ m. (L) Cumulative intensity histograms ($n = 30$) confirmed this disappearance of the podosome core fluorescence domain.

sion of WT OCs but also of WIP^{-/-} OCs adherent on glass or on HA. This suggested that the actin cloud is an important actor for osteoclast adhesion. Alternatively, glass adherent WIP^{-/-} OCs were insensitive to CD44 blocking antibodies, in contrast to WT or HA adherent WIP^{-/-} OCs, presenting podosome cores (Figure 5F). Together with the fact that WIP^{-/-} OCs devoid of podosome cores plated on glass were more sensitive than WT OCs to the $\beta 3$ integrin inhibitor (Figure 5F), we could conclude that the actin cloud, through $\beta 3$ integrin, is required for osteoclast adhesion, but this is strengthened by the presence of podosome cores and CD44.

In the search for a specific function for the actin cloud, we reasoned that myosin II, which is detected in the actin cloud in WT and WIP^{-/-} OCs (Figure 5, G and H), could play a role in OC contractility. To explore this hypothesis,

Figure 4. CD44 activation compensates WIP deletion by WASp stabilization. Localization and expression levels of three podosome cores markers, Arp3 (A–C), cortactin (D–F), and WASp (G–I) were analyzed in WT and WIP^{-/-} OCs seeded on glass slides by confocal microscopy and Western blot, respectively. In WIP^{-/-} OCs, Arp3 was expressed but diffusely distributed at the cell membrane; cortactin was confined to the actin cloud, and WASp was absent. In WIP^{-/-} OCs seeded on HA-coated slides, WASp (J) and Arp3 (K) were present in podosome cores. Western blot analysis confirmed the restoration of WASp levels in these OCs. Bar, 3 μ m.

an inhibitory strategy based on the pharmacological inhibition of myosin II was used. WT and WIP^{-/-} OCs were treated with blebbistatin and fixed (Figure 5, I–L). After 30-min exposure to the drug, cell spreading was evident. Quantitation of cell surface areas revealed that WIP^{-/-} and WT OCs were equally spread in the presence of blebbistatin (Figure 5M). Similar results were obtained with ML-7, a structurally unrelated inhibitor targeting myosin II ATPase activity (data not shown). These data show that WT and WIP^{-/-} OCs seeded onto serum-coated glass coverslips exhibit similar contraction status before the inhibitory treatment. OC contractility potential was next tested under more physiological conditions. In contrast to glass adhesion conditions, mature OCs seeded on apatite mineral are polarized and contracted. When WT and WIP^{-/-} OCs were seeded on ACC, a physiological and resorbable material, both cell types presented the same contraction status, as assessed by cell surface area measurements (Figure 5M). These data demonstrate that the sole presence of the actin cloud allows OC contractility, independently of podosome cores. This result strongly suggests that the actin cloud contributes to OC contractility necessary for the resorption process. We conclude that podosome cores and actin clouds have distinct functions in OC physiology, where podosome cores play a major role in adhesion and the actin cloud controls contractility.

CD44/WASp Is a Major Pathway for SZ Formation and Bone Resorption

Adhesion and contractility are crucial for the OC resorption process. Bone resorbing OCs exhibit a SZ, a large band of F-actin that does not result from the simple fusion of podosomes (Saltel *et al.*, 2004). Results presented so far suggested to us that the SZ could be derived from 1) the actin cloud only, 2) the reorganization of podosome cores only, or 3) podosome cores fusing with the actin cloud. To explore these possibilities, we first investigated how podosomal markers distributed in OCs seeded on ACC, a bone matrix mineralized in vitro containing apatite, the mineral part of bone. CD44 localized at the SZ in WT OCs but also in WIP^{-/-} OCs (Figure 6, A and C). These data show that the podosome core moiety is integrated in the SZ. Likewise, other specific components of podosome cores, such as cortactin and Arp3, were found in WIP^{-/-} SZ (data not shown). Confirming the results obtained on HA or OPN, WIP^{-/-} OCs seeded on ACC showed a classical SZ. WASp was detected at the SZ, and it appeared as in WT OCs (Figure 6, B and D). WASp expression under these conditions was also detected by Western blot (data not shown). We conclude that CD44 activation maintains WASp expression and localization at the SZ. Together, these data demonstrate the complexity of the SZ, which corresponds to the integration and reorganization of the two actin structures: podosome cores and actin clouds.

To assess the functionality of the SZ formed in WIP^{-/-} OCs, we compared the resorbing activity of WIP^{-/-} versus WT OCs. Mature OCs were seeded on ACC coverslips for 24 h, and then they were removed and the ACC coverslips were stained to quantify resorption. Data presented in Figure 6, E–G, show that WIP^{-/-} OCs were still able to resorb mineralized matrix but with a 30% lower efficiency than WT OCs. These data were confirmed on dentin substrates (data not shown). These results indicate that although apatite can activate CD44 and allows SZ formation, these signals do not compensate for the absence of WIP in functional assays. We conclude that the presence of WIP is indispensable for the formation of a fully functional SZ, necessary for bone resorption.

DISCUSSION

Fully differentiated multinucleated OCs exhibit two different actin structures, podosome belts and SZ, when adherent on artificial or apatite-mineralized substrates, respectively. In this study, we have extended our previous observation (Destaing *et al.*, 2003), recently confirmed by high-resolution scanning electron microscopy (Luxenburg *et al.*, 2007), that podosome belts are composed of two F-actin-containing domains, namely, a diffuse actin cloud surrounding actin dots referred to as podosome cores. The use of WIP-deficient OCs enabled us to define the molecular architectures of the two F-actin domains and to show for the first time that they fulfill different roles. The actin cloud is composed of proteins such as vinculin, paxillin, and signaling phosphoproteins, together with the α v β 3 integrin linking this domain to the ECM. In contrast, podosome cores are made of a dense F-actin network associated with proteins such as cortactin, Arp2/3, WASp, and the transmembrane receptor CD44 (Figure 7 and Table 1). As expected, WIP, which can interact directly with actin, WASp, and cortactin (Ramesh *et al.*, 1997; Martinez-Quiles *et al.*, 2001; Kinley *et al.*, 2003), and regulates podosome formation in dendritic cells (Chou *et al.*, 2006), colocalized with podosome cores. The potential role played by this protein in podosome formation was further emphasized by the analysis of WIP-deficient mature OCs; the actin

Figure 5. Podosome cores and actin clouds play different roles in OCs. Mature WT and WIP-/- OCs plated on glass were observed by confocal microscopy after F-actin staining (A and C) or by IRM (B and D). Bar, 6 μ m. Both cell types showed a large dark band at their periphery, corresponding to a tight attachment to the substratum, which perfectly colocalized with podosome belts. (E) Quantification of the adhesion strength of WT and WIP-/- mature osteoclast on glass was obtained after incubation of these cells with PBS/EDTA to induce their detachment. Remaining adherent osteoclasts were counted 0, 7, or 15 min after treatment. (F) To quantify adhesion strength, WT and WIP-/- mature OCs seeded on glass or on HA were incubated with CD44 blocking antibody (sc-7946) or β 3 blocking peptides (RGD). Remaining OCs were recorded 0, 20, or 30 min after treatment. Only adhesion of WT and WIP-/- OCs seeded on HA, which contained podosomes cores, decreased with sc-7946; in contrast, cell adhesion in the three cultures decreased with RGD peptides, demonstrating that both podosome cores and the actin cloud, and consequently the CD44 receptor and β 3 integrin subunits, participate in OC adhesion. Mature WT and WIP-/- OCs were plated on glass coverslips and observed by confocal microscopy after actin staining by phalloidin and immunolabeling for myosin II motor protein. Myosin II is localized in actin cloud WT (G) and WIP-/- (H) OCs. Bar, 4 μ m. (I-M) WT and WIP-/- OCs were plated on glass and treated with blebbistatin, an inhibitor of myosin II. Confocal microscopy after F-actin staining showed that the drug induced spreading of WT (I and J, dotted lines) and WIP-/- OCs (K and L, dotted lines), as indicated by pixel number. Bar, 60 μ m. (M) Quantification of cell spreading was achieved by measuring WT and WIP-/- mature OC surface areas, before and after the addition of 50 μ M blebbistatin (Bb), on glass or ACC slides. WIP-/- OCs have the same state and ability of contraction as WT OCs, demonstrating that the sole presence of the actin cloud allows OC contractility.

cloud domain remained, but no podosome cores could be observed by deconvolution confocal microscopy. In addition, WASp and Arp2/3 could not be detected, whereas cortactin redistributed within the cloud domain.

Comparisons between WT and WIP-/- OCs allowed us to define the role played by these two actin domains. Podosome cores strengthened adhesion to the substrate, because WIP-deficient OCs devoid of podosome cores still adhered to glass or plastic but much less effectively than WT OCs. We show that the actin cloud regulates OC contractility, in part through myosin II found in the cloud domain of WIP-/- OCs, because control and mutant OCs displayed similar contractility states when plated onto glass and similar contractility capacities when plated on mineralized substratum.

Podosome belts exhibit two transmembrane receptors with specific patterns, integrin α v β 3 underneath the actin

cloud and CD44 associated with podosome cores, whereas β 1 integrin could not be detected at the belt. Colocalization of CD44 with podosome cores is the first observation of a receptor distinct from integrins, directly associated with the actin core of podosomes. Podosome cores were reinduced in WIP-/- OCs seeded on HA, bringing new insight to understanding the role of HA in bone resorption (Spessotto *et al.*, 2002). Moreover, the use of blocking antibodies against CD44 induced podosome disassembly in WT OCs. Both α v β 3 integrin and CD44 are transmembrane receptors for several extracellular matrix elements, such as vitronectin for integrin α v β 3 and HA for CD44. One interesting point is that they share a common ligand, OPN, which is largely expressed and secreted by OCs (Chellaiah *et al.*, 2003b; Chellaiah and Hruska, 2003). These two receptors were presumed to play important functions in OC adhesion,

Figure 6. CD44/WASp is a major pathway for SZ formation and bone resorption. Mature WT and WIP-/- OCs were seeded on ACC coverslips. Confocal microscopy, after actin staining by phalloidin (insets) and immunolabeling for CD44 and WASp, showed that these proteins were present in the sealing zone of WT (A and B) and WIP-/- OCs (C and D). Bar, 10 μ m. After cell lysis and staining, resorbed surfaces areas by WT (E) and WIP-/- (F) OCs were observed under a stereomicroscope (MZ12; Leica). Bar, 200 μ m. Images were quantified (G) using MetaMorph and Photoshop software (see *Materials and Methods*). WIP-/- OC were still able to resorb mineralized matrix, but with a 30% lower efficiency than WT OCs (*t* test; *p* < 0.05).

migration, and resorption. Surprisingly, β 3 or CD44 null mice show only mild defects in bone resorption and only a weak osteopetrotic phenotype (McHugh *et al.*, 2000; de Vries *et al.*, 2005). Our results suggest that suppression of only one of these two receptors is not sufficient to block OC resorption. In contrast, OPN gene knockout induces a decrease in CD44 expression in OCs, which are hypomotile and less

Table 1. Composition of podosome cores

Component	WT OC podosome belt	WIP-/- OC podosome belt
Actin regulator		
WIP	Podosome cores	Absent
Arp2/3	Podosome cores	Absent
WAsp	Podosome cores	Absent
Cortactin	Podosome cores	Cloud
Integrin		
β 1	Absent	Absent
β 3	Cloud	Cloud
Other receptor		
CD44	Podosome cores	Absent
Integrin-associated protein		
Vinculin	Cloud	Cloud
Paxillin	Cloud	Cloud
Phosphorylated protein		
Phospho-tyrosine proteins	Cloud	Absent
Phospho-tyrosine paxillin	Cloud	Absent
Motors protein		
Myosin II	Podosome belt	Cloud

efficient in resorbing bone matrix (Chellaiah *et al.*, 2003b). Together these data suggest cooperativity between these two receptors in OCs. WIP-/- OCs seeded on coverslips previously coated by WT OCs exhibit podosome cores, suggesting that OCs secrete their own matrix to induce podosome core formation and subsequently bone resorption. Moreover, it can be anticipated that during the resorption cycle, the complex bone matrix underneath the OC will be altered as long as resorption progresses. Thus, a possible scenario could be the following: in an early phase of the process, OCs meet apatite, the mineral component of bone that is able to bind and concentrate matrix components such as OPN or HA (Reinholt *et al.*, 1990; Rees *et al.*, 2002). This high concentration of OPN or HA concentrates and activates CD44 to organize a SZ, a prerequisite for bone resorption. In the resorption lacuna delimited by the SZ, acidic dissolution of apatite resulting from H^+ secretion and proteolysis of the different bone matrix proteins, profoundly alters the bone matrix which can then promote the dissociation of the SZ and stimulate OC migration. This cycle could then be repeated.

Our present work has confirmed the essential role of WIP in podosome core formation. This molecule seems to act as a nucleator of actin-regulating proteins, because in its absence, OC podosome core components are completely dissociated, with delocalization of cortactin and Arp2/3. WIP has been shown to protect WASp from degradation via the proteasome or from proteolytic cleavage by calpain, in lymphocytes and dendritic cells (Chou *et al.*, 2006; de la Fuente *et al.*, 2007). In WIP-/- OCs, no specific staining for WASp was detected in the actin cloud, nor for Arp2/3 or phosphotyrosine proteins (Figure 7 and Table 1). However in the absence of WIP, the activation of CD44 by specific ligands such as HA, OPN, or laminin was sufficient to stabilize WASp and to reinduce podosome cores. Now, it will be interesting to determine precisely how CD44 restores WASp level, probably by protecting it from degradation. A direct CD44/WASp interaction could mask a site used by calpain, but CD44 could also inhibit ubiquitinylation of WASp and its degradation by the proteasome. This identification of CD44 as the first matrix receptor associated directly with the podosome core is consistent with its ability to interact with key elements involved in podosome core formation such as

Figure 7. Proposed schematic molecular organization of the two F-actin domains in OCs. Top left, detail of the cloud domain. A transversal F-actin network is nucleated via the $\alpha v\beta 3$ integrin and the multiprotein complex consisting of vinculin and paxillin. This complex is linked via talin and vinculin to a radial F-actin network. Only a few markers are represented for simplification purposes. Top right, detail of podosome core domain. A vertical F-actin network is nucleated at the membrane via the CD44 receptor and the actin polymerization machinery comprising WIP, WASp, cortactin, and Arp2/3. Bottom, schematic drawing of the F-actin belt in OC with an optical cut perpendicular to the substratum. The plasma membrane is delimited by an orange line. Adapted from Linder and Aepfelbacher, 2003 and Luxenburg *et al.*, 2007.

N-WASP, c-Src, and cortactin (Bourguignon *et al.*, 2001, 2007; Hill *et al.*, 2006).

Finally, the resorption capacity of WIP^{-/-} OCs was found to be slightly but significantly reduced compared with control OCs, even though the appearance of the SZ was normal. Thus, the clustering and activation of CD44 is sufficient to form a SZ, but the presence of WIP is necessary for the formation of a fully functional SZ. We can speculate that apatite allows WIP^{-/-} OC to bypass WIP deletion to some extent. This could be explained by the high-affinity of apatite for secreted matrix elements such as OPN or HA, which concentrate CD44 receptors and thereby trigger CD44 signaling pathways. How WIP contributes to the functionality of the SZ and its exact role in bone physiology are presently under investigation.

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