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The molecular dynamics of osteoclast adhesions

Chen Luxenburg[a], Lia Addadi[b], Benjamin Geiger[a,*]

[a]*Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel*
[b]*Department of Structural Biology, The Weizmann Institute of Science, Rehovot 76100, Israel*

Abstract

Podosomes are specialized adhesive structures that play a central role in bone resorption. In this article we address the molecular diversity and dynamics of podosomes at different states of organization, ranging from scattered distribution over the entire ventral membrane of non-polarized cells, via formation of podosome clusters and developing rings to the assembly of a peripheral belt, resembling the sealing zone of polarized, bone-resorbing osteoclasts. Based on published data and on our own results, we describe here the spatial relationships between key podosome-associated proteins. Using quantitative microscopy, we show here a dramatic increase in the local levels of F-actin, vinculin, paxillin, and α-actinin, which occurs upon the transformation of clustered podosomes into rings and sealing zone-like structures. This change is accompanied by a marked decrease in phosphotyrosine levels in the same region. Therefore, our data suggest that a major change in the molecular composition of podosomes is taking place during osteoclast polarization, a change that may be related to adhesion ''reinforcement'', associated with the assembly of the bone-resorbing apparatus. Studying the nature of the proteins that undergo de-phosphorylation is critical for the understanding of the mechanisms regulating the processes described above.

Keywords: Cell adhesion; Osteoclasts; Podosomes; Bone resorption; Sealing zone; Tyrosine phosphorylation

Introduction

Osteoclasts are multinucleated monocyte-macrophage derivatives that degrade bone, playing a central role in the formation, growth and remodeling of the skeleton. Excessive osteoclastic activity leads to progressive loss of bone mass, manifested by a variety of pathological conditions such as osteoporosis and common metastatic bone invasion. Reduced osteoclast activity, on the other hand, results in osteopetrosis, namely the formation of overly dense bone (reviewed in Blair, 1998; Rodan and Martin, 2000; Teitelbaum, 2000; Vaananen et al., 2000; Rodan, 2003). The fine physiological balance between bone formation and resorption is regulated at several distinct levels; osteoclast precursors need to home into the right skeletal sites, and differentiate into multinucleated cells that form a unique form of matrix adhesions known as podosomes (Marchisio et al., 1984; Zambonin-Zallone et al., 1988, 1989a). These adhesions undergo major reorganization during osteoclast maturation, eventually forming an adhesive apparatus at the cell periphery, known as the sealing zone. The sealing zone consists of a central actin belt surrounded by inner and outer rings of plaque proteins and integrins (Lakkakorpi et al., 1993).

The assembly can be readily monitored in cultured osteoclasts, where scattered podosomes give rise, sequentially, to podosome clusters, rings and finally to a stable belt of podosomes at the periphery of the cell (Destaing et al., 2003). The belt may further condense, leading to segregation of the plaque and the actin

*Corresponding author. Tel.: +972 8934 3910; fax: +972 8946 5607.
E-mail address: benny.geiger@weizmann.ac.il (B. Geiger).

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domains, giving rise to a sealing zone-like (SZL) structure. To avoid confusion, we will refer to the stable, peripheral structure of a mature osteoclast, plated on a conventional, non-degradable, glass or plastic substrate as SZL structure.

Podosomes are small, ~1 μm in diameter, dot-like adhesions that are found not only in osteoclasts, but also in other monocyte derivatives such as dendritic cells and macrophages and in various transformed cells. Recent reports suggest that certain smooth muscle cells and endothelial cells possess podosome-like structures as well (reviewed in Linder and Aepfelbacher, 2003). Structural analysis of podosomes in osteoclasts show that they consist of a cylindrical actin core, surrounded by a "ring" domain, enriched in $\alpha_v\beta_3$ integrin and plaque proteins, classically found in focal adhesions, including vinculin, paxillin, talin, tensin, and p130cas (Hiura et al., 1995; Lakkakorpi et al., 1993, 1999; Pfaff and Jurdic, 2001; Zambonin-Zallone et al., 1989b). Podosomes, unlike focal adhesions, also contain unique proteins such as matrix metalloproteases (Sato et al., 1997) and the tyrosine kinase PYK2 (Duong et al., 1998). In addition, podosomes are enriched with actin-associated proteins such as α-actinin, fimbrin (Marchisio et al., 1987), gelsolin (Chellaiah et al., 2000), cortactin (Hiura et al., 1995), actin-related protein complex 2/3 (arp 2/3) (Hurst et al., 2004), Wiskott–Aldrich Syndrome protein (WASp) (Calle et al., 2004), and the membrane invagination-associated protein dynamin (Ochoa et al., 2000).

Podosomes play a key role in the bone-resorptive activity of osteoclasts. Abnormal podosomes are associated with dysfunctional osteoclasts in several mice knockout models as well as in human osteopetrosis patients (Teti et al., 1999). Knockout for β_3 integrin leads to failure of osteoclasts to reorganize their cytoskeleton and to give rise to a sealing zone (McHugh et al., 2000), thus emphasizing the role of podosomes in signal transduction from the extracellular matrix. Similarly, deletion of the actin-capping protein gelsolin (Chellaiah et al., 2000), or the actin-nucleating protein WASp (Calle et al., 2004), blocks podosome assembly in osteoclasts.

The formation and transformation of adhesion structures is dynamically regulated by a wide variety of factors, including the chemical and physical nature of the substrate to which the cells attach (Katz et al., 2000; Balaban et al., 2001) and the forces applied to the cell adhesions (Riveline et al., 2001; Zaidel-Bar et al., 2005). Specific post-translational events, particularly tyrosine phosphorylation, are involved in the regulation of adhesion site assembly and modulate their molecular composition, development and dynamics (Kaplan et al., 1994; Volberg et al., 2001; Webb et al., 2004). This modification was shown to be critical for podosome formation, development and turnover (Tarone et al., 1985; Marchisio et al., 1987, 1988; Lakkakorpi et al., 2001). In line with these observations, it has been shown that in osteoclasts derived from mice lacking the non-receptor protein tyrosine kinase pp60c-src (src) (Soriano et al., 1991; Lakkakorpi et al., 2001) or the protein tyrosine phosphatase epsilon (Chiusaroli et al., 2004), podosomes are poorly organized and the cells cannot form a functional sealing zone.

Recent studies have indicated that while integrin-mediated adhesions and their association with the actin cytoskeleton are quite common, such adhesions may greatly vary in their subcellular location, size, molecular composition and dynamic properties (reviewed in Geiger et al., 2001). For example, focal complexes are small (~1 μm), stationary, short-lived, dot-like adhesions, located at the cell periphery. They can mature into elongated 2–5 μm-long structures, known as focal adhesions, which may further develop into fibrillar adhesion at the cell center. These are elongated (up to 10 μm) adhesions containing high levels of tensin and low levels of tyrosine phosphorylated proteins, relative to focal adhesions (Zamir et al., 1999; Zamir and Geiger, 2001; Zaidel-Bar et al., 2005).

The molecular diversity of podosomes and their dynamic properties have, so far, attracted limited attention, though it was shown that podosomes undergo dramatic reorganization during osteoclast polarization, from scattered podosomes to SZL super-structures. To date, however, the changes in the molecular composition during this process are poorly characterized. These are the aspects of podosome biology, which we would like to address here. Referring both to information derived from the literature and using pixel-to-pixel ratio analysis (Zamir et al., 1999) we describe here the relations between pairs of key podosomal proteins, as well as the increase in the local concentrations of actin, vinculin, paxillin, and α-actinin associated with the maturation of clustered podosomes into rings and ultimately into a mature SZL peripheral belt. We also show that a decrease in tyrosine phosphorylation is associated with the formation of the SZL structure.

Materials and methods

Antibodies

The primary antibodies used in this study included: rabbit anti-vinculin (R695; Geiger, 1979), rabbit anti-phosphotyrosine (PT40, kindly provided by Israel Pecht and Arie Licht, The Weizmann Institute), rabbit anti-phosphorylated (pY165) p130Cas (Cell Signaling Technology, Beverly, MA), rabbit anti-phosphorylated (pY118) paxillin (Biosource, Camarillo, CA), monoclonal antibody (mAb) anti-vinculin (clone hVin-1) and mAb anti α-actinin (clone BM-75.2) (Sigma Chemical Co., St Louis, MO, USA), mAb anti-paxillin and mAb

anti-p130cas (Transduction Laboratories, Lexington, KY, USA), and mAb anti-PY, #4G10 (Upstate Biotechnology, Charlottesville, VA, USA).

Secondary antibodies (all from Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) were: donkey anti-rabbit IgG, conjugated to cy5 and donkey anti-mouse IgG, conjugated to cy3 (both showing minimal cross-reactivity with Ig of other species, for multiple labeling) and goat anti-mouse IgG, conjugated to Cy3. F-actin was labeled with phalloidin conjugated to FITC (Sigma Chemical Co.).

Cells, fixation and staining

Osteoclast-like cells were prepared as described (Chiusaroli et al., 2004). Briefly, bone marrow cells from Balb/c mice were plated on a feeder layer of primary osteoblasts in 24-well plates, and cultured in alpha MEM with Earle's salts, containing L-glutamine and $NaHCO_3$ (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and antibiotics (Biological Industries, Beit Haemek, Israel), containing 10 nM 1,25-dihydroxyvitamin D3 (Sigma Chemical Co.) and 1 μM prostaglandin E2 (Sigma Chemical Co.) for 6–8 days.

RAW 264.7 cells were from the American Type Culture Collection (Manassas, VA, USA). To induce osteoclast differentiation, the cells were plated at a density of 100 cells/mm^2 in the medium described above, supplemented with 20 ng/ml recombinant soluble receptor activator of NF kappa B ligand (RANK-L) and 20 ng/ml macrophage colony-stimulating factor (R&D, Minneapolis, MN, USA), at 37 °C in a 5% CO_2 humidified atmosphere for 3 days.

For staining, cells were fixed for 2 min in warm 3% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) + 0.5% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA) and then in PFA alone for additional 40 min. After fixation cells were washed 3 times with PBS, pH7.4, and incubated with the primary antibody for 40 min, washed again 3 times in PBS and incubated for additional 40 min with the secondary antibodies.

Image acquisition and analysis

Microscopy-based data were acquired with a DeltaVision system (Applied Precision Inc., Issaquah, WA, USA), consisting of an inverted microscope IX70 (Olympus, Tokyo, Japan), using Resolve3D software.

Working on a Silicon Graphics workstation model O_2 (Mountain View, CA, USA) and using the Priism software package (www.msg.ucsf.edu/IVE), we produced ratio images between components, as previously described (Zamir et al., 1999). Briefly, ratio between components was calculated by dividing intensity of two components to provide a spectrum of numerators varying between 0.1 and 10.

Results

The molecular composition of podosomes

To visualize the molecular composition of podosomes and their reorganization during osteoclast polarization, murine bone marrow-derived cells were induced to differentiate into osteoclasts and labeled for pairs of actin-associated and ring domain-associated proteins. To best demonstrate the spatial relations between the different proteins, we have calculated spatial ratio images (Zamir et al., 1999). It is noteworthy that while the differential distributions of "core-associated" and "ring domain-associated" components have been extensively demonstrated in previous works (see, e.g. Zambonin-Zallone et al., 1989b; Lakkakorpi et al., 1993, 1999; Hiura et al., 1995; Pfaff and Jurdic, 2001), the use of accurate quantitative light microscopy and ratio imaging is capable of revealing differences in local intensities, presumably reflecting local concentrations of the different proteins. Thus, variations in the intensity ratio within an overlap area can indicate different organization states, reflecting variations in either the local levels of the protein pairs, or the accessibility to the labeling antibodies (Zamir et al., 1999). Double labeling for actin and either paxillin or vinculin (Fig. 1A and B) reveals the two domains in podosomes, namely, a paxillin- and vinculin-rich ring domain, surrounding an F-actin-rich core. This organization was apparent in both scattered podosomes and podosomes associated with the peripheral ring (Fig. 1A and B, right column). Double labeling for vinculin and paxillin indicated that the two proteins are largely colocalized in the ring domain (Fig. 1D). On the other hand, α-actinin, which colocalizes predominantly with actin in the podosome core (Fig. 1C), shows a wider distribution than actin and also partially colocalizes with the ring domain. This is indicated by the "yellow zone" in the vinculin–α-actinin ratio images (Fig. 1E, right). The spatial relations between the pairs of proteins described here are essentially identical to those found in podosomes of non-polarized osteoclasts (data not shown).

To further quantitatively address the molecular composition of podosomes, we compared the intensities of actin and of the various adhesion-related proteins in podosomes of non-polarized cells, to those associated with the peripheral SZL structure of polarized cells.

F-actin levels in podosomes scattered throughout the ventral membrane of non-polarized osteoclasts are 3- to 4-fold lower than those found in developing rings of podosomes (Fig. 2A, an arrowhead and an arrow indicate the respective structures). In addition, a further

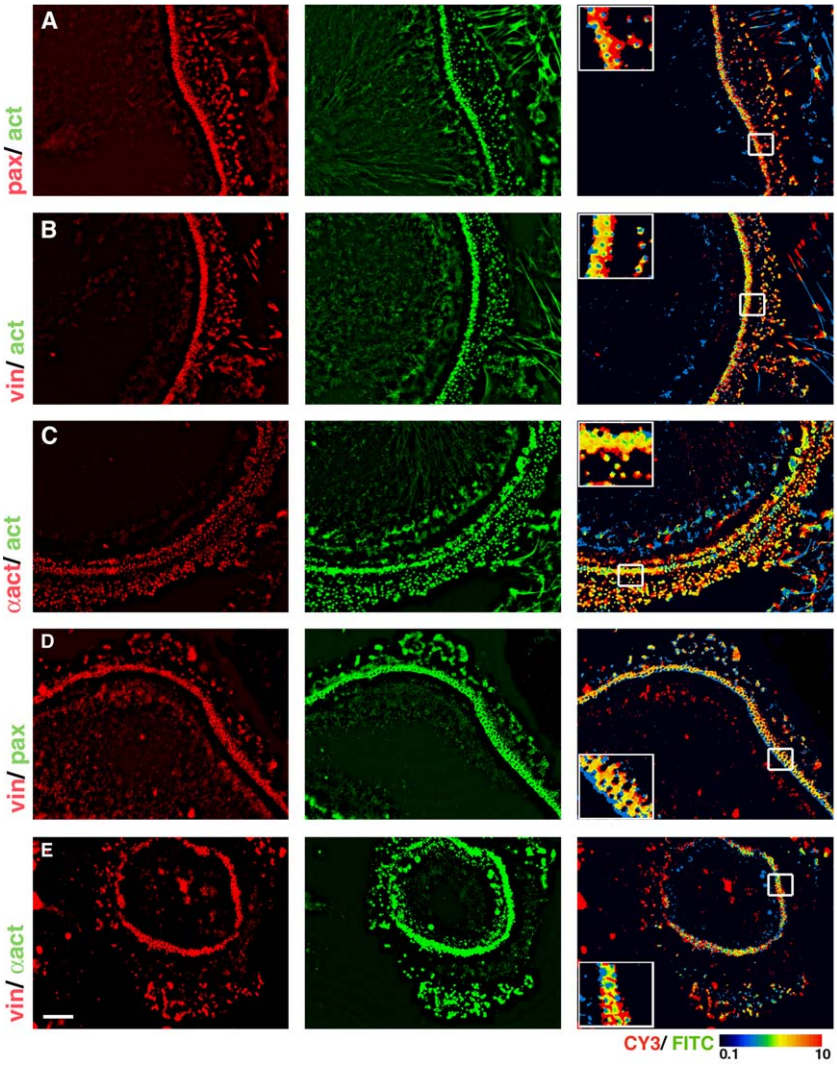

Fig. 1. Molecular domains of podosomes. Analysis of the differential distribution of pairs of podosome-associated proteins was assisted by calculation of pixel-to-pixel ratio images (right column) in cultured osteoclasts, derived from murine bone marrow. Paxillin and vinculin are associated with the podosome ring domain, surrounding the actin core (A and B, respectively). Vinculin and paxillin distributions are nearly overlapping (D). α-Actinin is largely co-localized with the actin core (C), yet its distribution is wider than that of actin, and it partially co-localizes with vinculin (E). Bar = 10 μm.

3- to 4-fold increase in F-actin intensity was noted in the SZL structure of polarized cells (Fig. 2A′). Interestingly, similar increases in intensity were noted for vinculin, paxillin and α-actinin (Fig. 2B and B′, C and C′, and D and D′, respectively). PY intensity, on the other hand, is highest in scattered podosomes both in non-polarized (Fig. 2E), and in polarized cells (Fig. 2E′, arrowhead), and is considerably lower in the SZL structure area (Fig. 2E′, arrow).

Differential tyrosine phosphorylation in non-polarized and polarized osteoclasts

The notion that tyrosine phosphorylation decreases in the SZL structure area of polarized cells, prompted us to study the relation between tyrosine phosphorylation and components of the core and of the ring domains of a podosome, in both polarized and non-polarized osteoclasts. The ratio images between PY and actin in non-polarized cells show that the actin cores are highly tyrosine-phosphorylated. It is, however, noteworthy that the phosphorylated area appears to be wider than the actin core proper (Fig. 3A). Similarly, scattered podosomes in polarized osteoclasts are phosphorylated along and beyond the actin core (Fig. 3B, inset 1) while actin cores of podosomes associated with the peripheral belt are not tyrosine-phosphorylated (Fig. 3A, inset 2). The ratio between paxillin and PY in non-polarized osteoclasts shows partial overlap, manifested by yellow-green pixels (Fig. 3C). This ratio further emphasizes the phosphorylation of the actin core (Fig. 3C, red pixels).

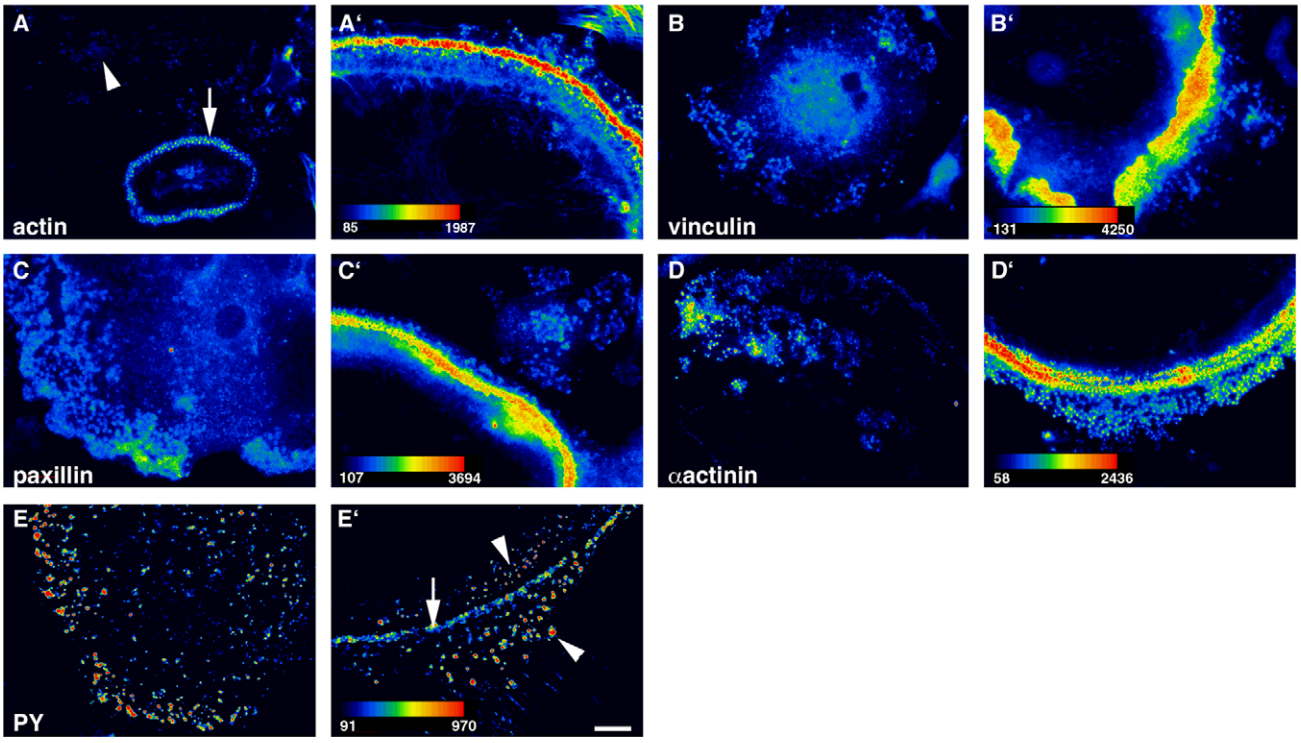

Fig. 2. Changes in the local intensities of podosome proteins in non-polarized and polarized osteoclasts. Pairs of polarized and non-polarized osteoclasts-like cells, derived from murine bone marrow were fixed, immunolabeled and examined by fluorescence microscopy. The following proteins were examined: F-actin (A,A′), vinculin (B,B′), paxillin(C,C′), α-actinin (D,D′), and tyrosine-phosphorylated proteins (PY) (E,E′). The intensity of F-actin, associated with podosomes, is ~3- to 4-fold higher in rings (arrow) relative to scattered podosomes (arrowhead) in non-polarized cells (A). In polarized cells the amount of actin associated with the SZL structure is even ~3- to 4-fold higher than in the rings (A′). Vinculin, paxillin and α-actinin intensities are also higher in the SZL belt of polarized cells relative to scattered podosomes present in the same cell or in non-polarized cells (B′,C′,D′ and B,C,D, respectively). PY in non-polarized cells is highest at the cell periphery (E); in polarized cells PY intensity is highest in scattered podosomes outside or inside the SZL structure (E′, arrowheads) whereas in the sealing zone PY levels are considerably lower (E′, arrow). Bar = 10 μm.

In the SZL structure of polarized cells, tyrosine phosphorylation associated with the paxillin ring domain is similar to that found in non-polarized cells, but the actin core is clearly non-phosphorylated (Fig. 3D).

To study tyrosine phosphorylation of specific proteins in the podosome ring domain, we fixed and triple stained cultured RAW-derived osteoclasts for paxillin, phospho-paxillin (pY118) and actin or p130cas, phospho-p130cas (pY165) and actin (Fig. 4). The paxillin/actin and p130cas/actin ratio images indicate that the two proteins are localized in the podosome ring domain (Fig. 4 "pax/act and cas/act"). The ratio between paxillin/paxillin pY118 shows that paxillin phosphorylation on Y118 is limited to a small fraction of the total paxillin, mostly to paxillin along the inner plaque ring (Fig. 4 "pax/pax pY118"). The p130cas/p130cas pY165 ratio reveals partial phosphorylation in the outer plaque ring while the inner plaque ring is intensely phosphorylated (Fig. 4 "cas/caspY165").

We conclude that a dramatic change in phosphorylation of the two podosome domains is taking place during reorganization of podosomes from scattered organization to the SZL structure. Moreover, the phosphorylation of the same plaque proteins may vary according to their subcellular localization.

Discussion

The molecular composition of adhesion structures is highly dynamic and diversified. Adhesion-related proteins enter and exit adhesion structures in a sequential manner according to various signals (Zaidel-Bar et al., 2003), hence changing the molecular properties of the adhesion sites. For example, during the maturation of focal complexes into focal adhesions and the later segregation into fibrillar adhesions, there are major changes in the molecular composition of the adhesion sites, which can affect the functional properties of the respective sites, as well as their subcellular distribution and mode of interaction with the cytoskeleton (Zamir et al., 1999, 2000). Our primary objective, in this article, is

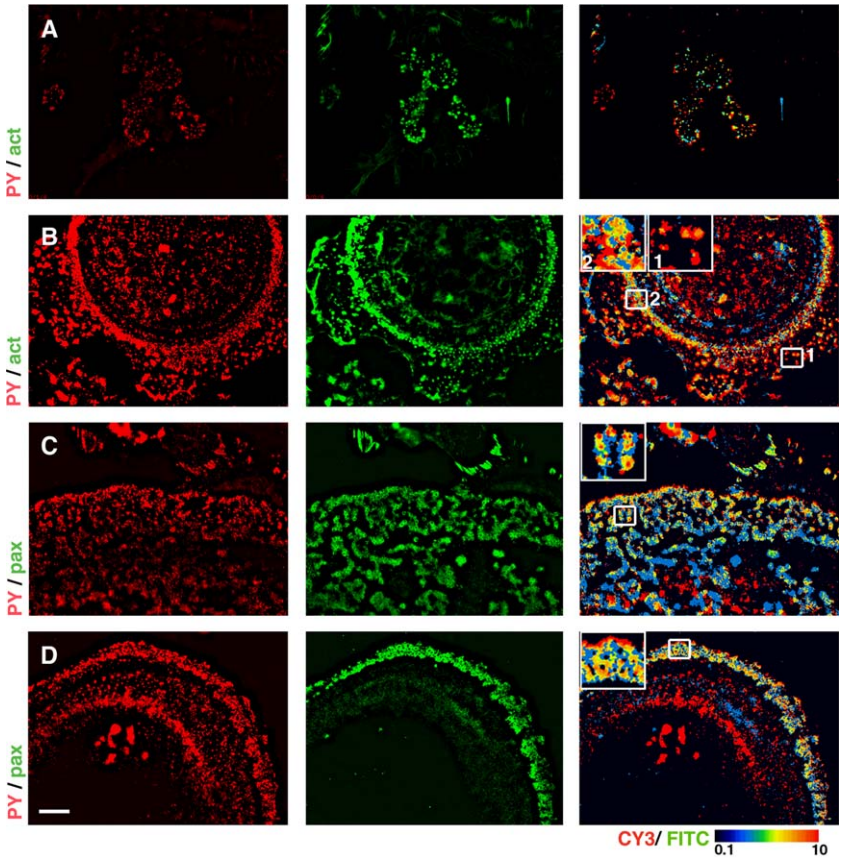

Fig. 3. Analysis of podosome-associated tyrosine phosphorylation in polarized and non-polarized osteoclasts. Osteoclasts derived from murine bone marrow were fixed and double stained for PY and actin (A, B) or PY and paxillin (C, D) and analyzed by calculation of pixel-to-pixel ratios (right column). The actin cores of scattered podosomes in non-polarized cells are phosphorylated (A). In polarized cells actin of clustered podosomes is phosphorylated whereas sealing zone-associated actin is not (B, ratio insets 1 and 2, respectively). Notice that double labeling for paxillin and PY demonstrates the same phenomenon (C, scattered podosomes and D, SZL). Bar = 10 μm.

to address the molecular diversity of a very special type of integrin-mediated adhesions, namely podosomes in developing osteoclasts. Podosome molecular composition was extensively studied in recent years, yet its modification during osteoclast polarization is still poorly understood. Podosomes contain two major structural domains, namely, an actin-rich core and a ring surrounding it. Moreover, during osteoclast polarization, podosomes undergo transformation into different types of super-structures. Specifically, individual podosomes, scattered throughout the ventral cell surface, tend to cluster into well-organized arrays which further develop into ring-like structures, and eventually into a continuous SZL belt. While the structural building blocks of all the adhesions formed in osteoclasts are apparently the same, namely the ''basic podosome'', it is not clear whether the reorganization of podosomes is accompanied by, or even driven by specific changes in the molecular composition of these structures. Recently, Saltel et al. (2004) plated osteoclasts on a degradable matrix, and observed the formation of a ''de-novo sealing zone'' expanding towards the periphery of the cell and eventually developing into a mature sealing zone. Podosomes were not observed to form. However, reports from other laboratories, based on plating of osteoclast on bone or dentin slices, do report the presence of podosomes as well as of a sealing zone (Zambonin-Zallone et al., 1988; Lakkakorpi et al., 1993; Calle et al., 2004). The reason for the contradictory reports may arise from the different sources of osteoclasts or from differences in the origin and preparation of the matrices. All the reports described above, however, suggest the involvement of the same plaque- and actin-associated proteins in the different structures, thus emphasizing the need to understand differences at the molecular level between the different adhesion structures.

The use of quantitative microscopy enabled us to accurately examine, not just the spatial distribution of the various podosomal proteins, but also their fluorescence intensity levels. These measurements revealed a dramatic increase in the labeling of podosomes for actin,

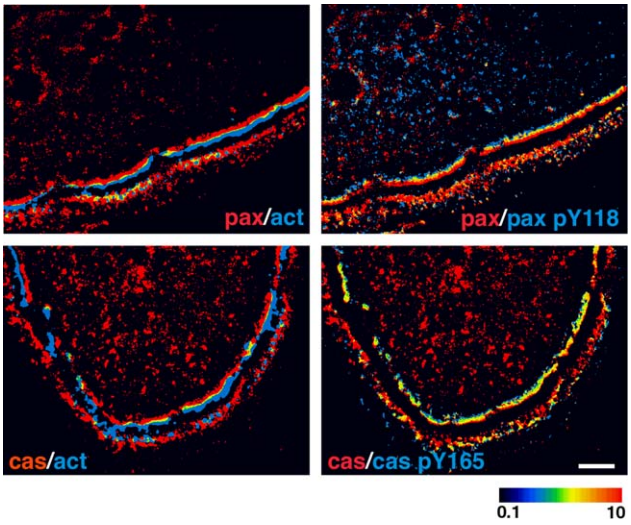

Fig. 4. The spatial distribution of tyrosine-phosphorylated paxillin and p130cas in polarized osteoclasts. Osteoclasts derived from RAW 264.7 cells were induced to differentiate and triple stained for paxillin, paxillin (pY118) and actin or for p130cas, p130cas (pY165) and actin. Both paxillin and p130cas localize to the plaque domain flanking the SZL structure (pax/act and cas/act). Paxillin phosphorylation on Y118 is moderate and enriched in the inner plaque ring (pax/pax pY118), while p130cas phosphorylation on Y165 is dramatically enriched in the inner plaque ring (cas/cas pY165). Bar = 10 μm.

vinculin, paxillin and α-actinin, which occurs concomitantly with the formation of rings of podosomes upon cell polarization. These changes reflect a major increase in the levels of the respective proteins, which is, apparently, regulated locally, judging from the fact that podosomes associated with the SZL structure contain higher levels of these proteins, relative to scattered podosomes located near by within the same cell. These data suggest that the elevated levels of actin are accompanied (or even preceded) by the formation of a thicker or denser plaque. These elevated amounts may reflect re-organization of the podosomes, which might be essential for the assembly of the sealing apparatus of a bone-resorbing osteoclast.

Another difference between scattered podosomes and podosomes associated with the peripheral SZL belt is the level of tyrosine phosphorylation within the core and the surrounding plaque. This post-translational modification plays a key role in adhesion development (Volberg et al., 2001) and dynamics (Webb et al., 2004). Moreover, it was shown that excessive tyrosine phosphorylation induced by overexpression of a deregulated src, can lead to the formation of podosomes in fibroblastic cells (Tarone et al., 1985). Here we report that phosphorylation associated with the actin cores in the SZL structure of polarized cells is lower than that found in scattered podosomes in the same cell or in non-polarized cells. The identity of the protein(s) showing modulated phosphorylation during podosome maturation is currently under investigation, motivated by the hypothesis that these molecules, as well as the kinases and phosphatases that regulate their phosphorylation, play essential roles in the podosome development during osteoclast polarization, and also in the assembly of podosomes into a functional resorptive apparatus.

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