Abnormal localisation and hyperclustering of αvβ3 integrins and associated proteins in Src-deficient or tyrphostin A9-treated osteoclasts

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SUMMARY

The non-receptor tyrosine kinase Src was shown to be essential for osteoclast function in vivo. We have previously reported that engagement of αvβ3 integrin in osteoclasts induces tyrosine phosphorylation and activation of the adhesion kinase Pyk2 and the adaptor protein P130Cas in a Src-dependent manner. The objective of this study was to analyse the role of c-Src in the αvβ3 integrin-dependent recruitment of signalling and cytoskeletal molecules in osteoclasts during bone resorption. Using prefusion osteoclasts (pOCs) obtained from cocultures of osteoblasts and spleen cells isolated from Src−/− mice or their normal littermates, we found: (1) similar expression levels and ligand binding affinities of αvβ3 integrins in Src−/− and Src+/+ pOCs, (2) reduced adhesion and spreading of Src−/− pOCs, (3) defective organisation of the microfilament proteins, F-actin, vinculin and paxillin, and of Pyk2 and P130Cas in the sealing zone of Src+/− OCLs, and (4) hyperclustering of αvβ3 integrins together with microfilament and signalling proteins in the basal membrane of Src-deficient OCLs. In normal OCLs, the tyrosine kinase inhibitor tyrphostin A9 inhibits actin ring formation, bone resorption and tyrosine phosphorylation of several proteins, including c-Src. Furthermore, tyrphostin A9 induced similar hyperclustering of αvβ3 integrins in osteoclasts as observed in Src−/− OCLs. Taken together, these findings suggest that normal localisation of αvβ3 and recruitment of its downstream effectors to the appropriate compartments of the osteoclast during resorption depend on Src kinase activity.

Key words: Osteoclast, αvβ3 integrin, c-Src, Polarisation

INTRODUCTION

Osteoclasts are highly differentiated bone resorbing cells of hematopoietic origin. Bone resorption is a multistep process, initiated by osteoclast adherence to bone matrix leading to cytoskeletal reorganisation, cellular polarisation and formation of unique membrane areas for bone resorption (Suda et al., 1997; Roodman, 1999). During resorption osteoclastic microfilaments form a specific ring structure, called the sealing zone, which mediates tight attachment of the cell to mineralised bone matrix (Lakkakorpi et al., 1989; Lakkakorpi and Viäänen, 1991). The sealing zone surrounds a convoluted membrane area, called the ruffled border, formed as a result of active, directional secretion. Protons and proteases secreted into the compartment between the cell and the bone matrix dissolve mineral and organic components of bone (see review in Chambers and Hall, 1991). During active resorption, degraded bone matrix is endocytosed into the osteoclast, transported through the cell and secreted at the basal plasma membrane facing bone marrow (Nesbitt and Horton, 1997; Salo et al., 1997).

The non-receptor tyrosine kinase c-Src has been shown to be essential for osteoclast function, since targeted disruption of c-Src in mice caused osteopetrosis (Soriano et al., 1991). The number of osteoclasts appears to be normal in Src-deficient mice, but these cells are not able to form ruffled border, which leads to impaired bone resorption in vivo and in vitro (Lowe et al., 1993). In addition, c-Src is largely localised to the ruffled border membrane and to vacuoles in osteoclasts (Boyce et al., 1992; Tanaka et al., 1992). αvβ3 integrin, which is the major integrin in osteoclasts, is also important for osteoclast function. Targeted disruption of β3 integrin in mice induces progressive osteosclerosis without an apparent reduction in osteoclast number (McHugh et al., 2000). αvβ3 integrin is suggested to mediate the initial adhesion of osteoclasts to bone matrix and to be necessary for osteoclast migration and progression of bone resorption (Davies et al., 1989; Sato et al., 1990; Lakkakorpi et al., 1991; Nakamura et al., 1999). Engagement of αvβ3 integrin in osteoclasts, induces tyrosine phosphorylation of Pyk2 (also known as Cakβ, Raftk or Cadtk), a member of the focal adhesion kinase family, and of the adaptor protein P130Cas (Duong et al., 1998; Lakkakorpi et al., 1999). Both Pyk2 and P130Cas localise in the sealing zone in resorbing osteoclasts. Activation and tyrosine phosphorylation of Pyk2 and P130Cas are Src-dependent, since their tyrosine phosphorylation is markedly reduced in osteoclasts derived from Src−/− mice (Duong et al., 1998; Nakamura et al., 1998; Lakkakorpi, 1999). Furthermore, Pyk2 activity is reduced when osteoclasts are treated with bone resorption inhibiting agents, such as ecalstatin, calcitonin and cytochalasin D.
(Duong et al., 1998). In addition, the proto-oncogene c-Cbl was found to be tyrosine phosphorylated in an Src-dependent manner in osteoclasts and thus suggested to be downstream in the Src signalling pathway required for bone resorption (Tanaka et al., 1996).

The objective of this study was to examine the role of Src tyrosine kinase in the \(\alpha_\beta_3\) integrin-mediated recruitment of downstream signalling and structural molecules in osteoclasts seeded on bone matrix. We show that the expression level and ligand binding affinity of \(\alpha_\beta_3\) integrins are similar in Src-deficient and wild-type osteoclasts. However, during in vitro resorption, the lack of c-Src results in pronounced aggregation of deficient and wild-type osteoclasts. However, during in vitro resorption, the lack of c-Src results in pronounced aggregation of the same molecules. Taken together, these data support a crucial role for c-Src in the dynamic interactions, between \(\alpha_\beta_3\) integrins and their downstream effectors, which are important for osteoclast polarisation during bone resorption.

**MATERIALS AND METHODS**

**Reagents**

Tissue culture media were purchased from Gibco (Grand Island, NY, USA), fetal bovine serum (FBS) from JRH Bioscience (Lenexa, KS, USA), collagenase from Wako Chemicals USA (Richmond, VA, USA) and dispase from Boehringer Mannheim (Mannheim, Germany). 1\(\alpha\),25-dihydroxyvitamin D\(_3\) [1\(\alpha\),25(OH)\(_2\)D\(_3\)] was a gift of Dr Milan R. Uskokovic (Hoffman-La Roche Inc., Nutley, NJ, USA). Echinostatin and 12\(\alpha\)-echistatin were generated as described (Nakamura et al., 1998). Anti-\(\beta_3\) and anti-\(\alpha_\beta_3\) monoclonal antibodies were from Pharmingen (San Diego, CA, USA). Anti-PYK2 and anti-\(\beta_3\) polyclonal antibodies were as previously described (Duong et al., 1998). Anti-p130Cas polyclonal antibodies were a gift of Dr J. Thomas Parsons (University of Virginia, Health Science Center, Charlottesville, Virginia, USA). Anti-p130Cas (mAb 21) and anti-paxillin (mAb 349) antibodies were from Transduction Labs (Lexington, KY, USA), and anti-vinculin (mAb V11-5) from Sigma. Anti-pp60\(^{Src}\) (mAb GD11) and antiphosphotyrosine (mAb 4G10) were from Upstate Biotech (Lake Placid, NY, USA). FITC- and TRITC-conjugated IgGs were from Jackson Labs (West Grove, PA, USA), horseradish peroxidase-conjugated IgG from Amersham (Arlington Heights, IL, USA) and Oregon Green 488 phalloidin from Molecular Probes Inc. (Eugene, OR, USA). Protein kinase inhibitors tyrphostin A9 and AG126 were from Biomol Res. Labs (Plymouth Meeting, PA, USA).

**Animals**

Heterozygous Src\(^{+/−}\) mice were obtained from Jackson Labs (Bar Harbor, ME, USA) and mated to generate osteopetrotic Src\(^{-/−}\) mice, which were identified by the lack of tooth eruption. All animals were cared for according to Institutional Animal Care and Use Committee (IACUC) Guide.

**Cell cultures**

Prefusion osteoclasts (pOCs) were prepared as described previously (Wesolowski et al., 1995). Briefly, Src\(^{+/−}\) and Src\(^{++/−}\) fusion osteoclasts were obtained from cocultures of osteoblastic MB1.8 cells and spleen cells isolated from 2–3 week old Src\(^{+/−}\) mice and their normal littersmates, respectively. After 5 days in coculture in \(\alpha\)MEM containing 10% FBS and 10 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\), pOCs were released from dishes with 10 mM EDTA in Ca\(^{2+}\)/Mg\(^{2+}\)-free PBS after removing MB1.8 cells with collagenase and dispase (0.1% each in PBS). Alternatively, cocultures were kept for 7–8 days to generate multinucleated osteoclast-like cells (OCLs) and purified as described previously (Duong et al., 1998).

**Assays**

Formation of actin rings and resorption pits were determined as previously described (Suda et al., 1997). To determine the number of actin rings, samples (1000 cells/well) of OCLs were plated onto 96-well plates with graded concentrations of tyrphostin A9 or AG126 for 4 hours. Cells were then fixed in 4% paraformaldehyde and double-stained for tartrate-resistant acid phosphatase (TRAP), a marker enzyme for osteoclasts, and F-actin. Numbers of TRAP-positive cells and of cells with actin rings were counted and the data were expressed as means ± s.d. (\(n=4\) per condition). For pit formation assay, OCLs were plated on dentine slices in 96-well culture plates. After 4 hours, dentine slices were transferred into 24-well plates with various concentrations of tyrphostins. After 20 hours, pit numbers and area were measured using an image analysing system (Empire Imaging Systems, Milford, NJ, USA). The data were expressed as percentage of resorbed area per dentine surface area (\(n=5\) per condition).

**Immunoblotting and immunoprecipitation**

Src\(^{+/−}\) and Src\(^{++/−}\) pOCs were lysed into modified RIPA buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.2% sodium deoxycholate, 1 mM EDTA, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 10 \(\mu\)g/ml aprotinin and 10 \(\mu\)g/ml leupeptin) on ice. Lysates containing the same amounts of proteins were precleared with Sepharose 4B and precipitated with anti-\(\alpha_\beta_3\) integrin, anti-Src, anti-PYK2 or anti-paxillin antibodies for 1 hour at 4°C, followed by G-protein-Sepharose for 1 hour at 4°C, and finally washed with RIPA buffer (4×). Total cell lysates or immunoprecipitated proteins were separated by 4%-12% gradient, 8% or 12% SDS-PAGE (Novex, San Diego, CA, USA), electrottered onto Immobilon-P membrane (Millipore, Bedford, MA, USA), which was incubated subsequently with primary antibodies, followed by HRP-conjugated secondary antibodies, and then developed with an enhanced chemiluminescence system (ECL, Amershams Pharmacia Biotech).

**Binding and adhesion assays**

The same number of Src\(^{+/−}\) and Src\(^{++/−}\) pOCs were resuspended in \(\alpha\)MEM and 0.1% BSA. Cells (5,000 cells per condition) were then incubated with 30 pM 12\(\alpha\)-echistatin for 1 hour at 4°C, in the presence of increasing concentrations of unlabeled echistatin. Bound and unbound radiolabeled echistatin were separated using a semiautomatic cell harvester (Skatron Inc., Sterling, VA, USA) and washed with PBS. Bound radioactivity was determined using a Cobra II auto-gamma counter (Packard Inst. Co., Meriden, CT, USA). In the adhesion assay, Src\(^{+/−}\) and Src\(^{++/−}\) pOCs were allowed to attach to vitronectin-coated dishes (20 \(\mu\)g/ml, Life Technologies Inc.) at the indicated times in the absence of serum. After gently washing (twice) in Hanks’ balanced salt solution to remove non-adherent cells, attached cells were fixed, stained for TRAP, and counted using an image-analysis system (empire Image System, Milford, NJ, USA).

**Immunofluorescence**

Src\(^{+/−}\) and Src\(^{++/−}\) OCLs cultured on bone slices together with 1,25(OH)\(_2\)D\(_3\)-pretreated MB1.8 cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. In studies using tyrophostins, OCLs were first allowed to adhere to bone slices and to start bone resorption for 20 hours, then treated with tyrophostins for 4 hours, followed by fixation and immunostaining. Cells were stained using polyclonal anti-\(\alpha_\beta_3\), anti-PYK2, poly- or monoclonal anti-p130Cas, anti-vinculin and anti-paxillin antibodies, followed with the appropriate secondary antibodies, or with Oregon green.
Green 488 phalloidin. Stainings were viewed with a Leica TCS SP Spectral confocal laser scanning microscope equipped with Argon-Krypton laser (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

RESULTS

Reduced adhesion and spreading of Src-deficient pOCs, without changes in the expression level or binding affinity of \( \alpha_\nu \beta_3 \) integrins

Since both c-Src and \( \alpha_\nu \beta_3 \) integrins were previously shown to be rate limiting in osteoclast function (Soriano et al., 1991; McHugh et al., 2000), we examined the effects of Src deficiency on \( \alpha_\nu \beta_3 \) integrin-mediated cytoskeletal rearrangement during cell adhesion and spreading, using Src\(^{+/}\)prefusion osteoclast-like cells (pOCs). Src\(^{-/-}\) and Src\(^{+/}\) pOCs were allowed to adhere to vitronectin-coated dishes for the indicated times in the absence of serum. The number of attached cells was counted after TRAP staining. Adhesion kinetics for Src\(^{-/-}\) pOCs appeared to be slower than for Src\(^{+/}\) pOCs (Fig. 1A), and after 1 hour the number of attached Src-deficient cells was significantly lower (65%) than that of Src\(^{+/}\).

![Fig. 1. Reduced adhesion and spreading of Src-deficient pOCs.](image)

Fig. 2. Expression level of \( \alpha_\nu \beta_3 \) integrins is similar in Src\(^{+/}\) and Src\(^{-/-}\) pOCs. (A) Equal amounts of proteins from total cell lysates (TCL) of Src\(^{+/}\) and Src\(^{-/-}\) pOCs were separated by 12% SDS-PAGE, transferred onto Immobilon-P, and blotted with both anti-\( \beta_3 \) integrin and anti-Src antibodies, followed by reblotting with anti-\( \alpha_\nu \) integrin and anti-Src antibodies. The molecular markers (kDa) are indicated on the left. (B) TLCs from Src\(^{+/}\) and Src\(^{-/-}\) pOCs were immunoprecipitated with anti-\( \alpha_\nu \) integrin antibodies and blotted with anti-\( \beta_3 \) integrin antibodies followed by anti-\( \alpha_\nu \) integrin antibodies. Similar TLCs were also immunoprecipitated and probed for Src.
cells, probably due to impaired spreading (Fig. 1B), resulting in cell loss during washing.

Since \( \alpha_v \beta_3 \) is the major integrin in osteoclasts and its engagement by ligand results in recruitment of c-Src into the adhesion-dependent signalling complexes, we next analysed the expression and binding affinity of \( \alpha_v \beta_3 \) integrin in pOCs from Src\(^{-/-}\) and Src\(^{+/+}\) mice. Western blotting and immunoprecipitation experiments revealed that the expression of both \( \alpha_v \) and \( \beta_3 \) subunits are similar in Src\(^{-/-}\) and Src\(^{+/+}\) pOCs (Fig. 2). Furthermore, the binding affinities of \( \alpha_v \beta_3 \) integrins to radiolabelled echistatin ligand were also similar in Src\(^{-/-}\) and Src\(^{+/+}\) pOCs, yielding IC\(_{50}\) values of 0.3-0.4 nM echistatin, respectively (Fig. 3). The data suggest that a change in \( \alpha_v \beta_3 \) abundance or properties is not responsible for the differences in adhesion between Src\(^{-/-}\) and Src\(^{+/+}\) pOCs.

Defective organisation of the sealing zone in Src-deficient osteoclast-like cells on bone matrix

Since the expression and ligand binding affinities of \( \alpha_v \beta_3 \) integrins are similar in Src\(^{-/-}\) and in Src\(^{+/+}\) osteoclasts and antagonists of these integrins cause disruption of the sealing zone in resorbing osteoclasts (Nakamura et al., 1999), we examined the distribution of several proteins which have a characteristic localisation during osteoclast polarisation on bone. As previously reported (Lakkakorpi et al., 1991; Masarachia et al., 1995; Duong et al., 1998; Lakkakorpi et al., 1999; Lakkakorpi et al., 1989), in wild-type osteoclast-like cells (OCLs) during resorption \( \alpha_v \beta_3 \) integrins localise to the basal membrane, to intracellular vesicles and to the ruffled border, while PYK2, p130Cas and cytoskeletal proteins including F-actin, vinculin and paxillin are primarily found in the sealing zone. Using confocal microscopy, we confirmed localisation of \( \alpha_v \beta_3 \) integrins in the basal membrane and ruffled border (Fig. 4Ab,c,g, in red) and localisation of cytoskeletal molecules (Fig. 4Aa,c,g, Fig. 4B,a,b, in green) as well as PYK2 (Fig. 5a,c, in red) in the sealing zone. Surprisingly, Src\(^{-/-}\) OCLs have sealing zone-like structures at the bone surface, which appear to be podosomal adhesion contacts containing F-actin, paxillin and vinculin, organised into ring-like structures (Fig. 4Ad,h,bc,d, in green). In fact, the ring-like adhesion structures in Src\(^{-/-}\) OCLs were similar to intermediate stage sealing zones prior to being fully formed, previously characterised in normal osteoclasts (Lakkakorpi and Väänänen, 1991). An example of the intermediate or immature sealing zone in a wild-type OCL is shown in Fig. 4Bb, in green. Src\(^{-/-}\) OCLs (Fig. 4Bc,d) never formed the double circle structure containing paxillin (seen in Fig. 4Ba, in green), a characteristic feature of mature sealing zones found in normal resorbing osteoclasts (Lakkakorpi and Väänänen, 1991). In agreement with previous observations (Lakkakorpi and Väänänen, 1991), we found that vinculin has the same localization as paxillin in the sealing zone of osteoclasts (data not shown). In addition, PYK2 (Fig. 5d,f) or p130Cas (data not shown) appeared to be infrequently localised in the sealing zone-like structures of Src-deficient OCLs.

To further characterise the sealing zone-like structures of Src\(^{-/-}\) OCLs, we coimmunostained and quantified the presence of paxillin, PYK2 or p130Cas in the actin-rich ring structures on bone. We found that paxillin always colocalised with F-actin. In contrast, PYK2 was found only in 43% and p130Cas in 33% of these intermediate sealing zone structures containing F-actin. In control OCLs under the same conditions, colocalization frequency of paxillin, PYK2 and p130Cas with F-actin were 98%, 84% and 83%, respectively. Interestingly, \( \alpha_v \beta_3 \) integrins, which are not normally found in mature sealing zones of osteoclasts, colocalised with 16% of these F-actin-containing intermediate sealing zone structures (Fig. 6). These results suggest that the progression of sealing zone formation is impeded in Src-deficient osteoclasts during the polarisation process.

Hyperclustering of \( \alpha_v \beta_3 \) integrins in basal membrane of Src-deficient osteoclast-like cells on bone matrix

In contrast to the fairly even distribution of \( \alpha_v \beta_3 \) integrins in the basal membrane (Fig. 4Ag, in red), around the leading edge and at the ruffled border (Fig. 4Ab) in wild-type OCLs during resorption, confocal microscopy revealed that in Src\(^{-/-}\) OCLs, \( \alpha_v \beta_3 \) integrins appeared to cluster prominently to 5-10 \( \mu \)m size patches, at the basal surface of OCLs (Figs 4Af,h,bc,d, 7b-f, in yellow). It should be noted that in control OCLs, no obvious selective localisation of vinculin, paxillin or p130Cas was observed at the basal plasma membrane. This suggests that the lack of c-Src results in hyperclustering of \( \alpha_v \beta_3 \) integrins and recruitment of its signalling and cytoskeletal partners to the basal membrane of osteoclasts. In addition, hyperclustering of \( \alpha_v \beta_3 \) integrins occurred concomitantly with incomplete organisation of the sealing zones in Src-deficient osteoclasts during polarisation. This suggests that c-Src might play an important role in the dynamic organisation of \( \alpha_v \beta_3 \)-dependent downstream cytoskeletal and signalling molecules, involved in sealing zone formation.
Fig. 4. Localization of \( \alpha_v\beta_3 \) integrins and of microfilaments in resorbing control OCLs and in Src-deficient OCLs on bone. (A) Pseudocolored confocal microscopic images show localisation of F-actin (a,d,g,h, in green) and \( \alpha_v\beta_3 \) (b,e,g,h, in red) in control (a-c,g) and Src\(^{-/-} \) OCLs (d-f,h). Colocalisation is seen as yellow in overlay images (c,f,g,h). Note more irregular F-actin rings (arrows, d) and \( \alpha_v\beta_3 \) patches (e) in Src\(^{-/-} \) OCLs compared to F-actin rings at the sealing zone (arrows, a) and \( \alpha_v\beta_3 \) at the ruffled border (b) and basal plasma membrane (g) in control OCLs. Note also colocalisation of \( \alpha_v\beta_3 \) with microfilaments in basal patches in Src-deficient OCLs (arrowheads, f). In a vertical section, F-actin is found at the bone surface (arrow in g) and \( \alpha_v\beta_3 \) mainly at the basal surface in control OCLs (g), whereas in Src\(^{-/-} \) OCLs, F-actin is at the bone surface (arrow, h) and colocalising with \( \alpha_v\beta_3 \) at the basal patches (arrowhead, h). (B) Localisation of \( \alpha_v\beta_3 \) (a,c,d, in red), paxillin (a-c, in green), and vinculin (d, in green) in control (a,b) and Src\(^{-/-} \) OCLs (c,d) is shown. In control cells, paxillin localises as a double circle at the sealing zone (a, arrows) or as a broad ring in the immature sealing zone (b, arrows), whereas Src\(^{-/-} \) OCLs have only immature sealing zone structures, containing paxillin (c, arrows) or vinculin (d, arrows). Note also colocalisation of \( \alpha_v\beta_3 \) with paxillin and vinculin at the basal patches in Src\(^{-/-} \) OCLs (c,d, arrowheads). Merged images from optical sections from 13.2 \( \mu m \) (A,a-c), 7.3 \( \mu m \) (A,d-f), 3.6 \( \mu m \) (B,a), 5.4 \( \mu m \) (B,b), 3.1 \( \mu m \) (B,c) and 2.6 \( \mu m \) (B,d) thickness close to the bone surface are shown. Bars, 10 \( \mu m \).
Tyrphostin A9 inhibits tyrosine phosphorylation of c-Src, PYK2 and paxillin in osteoclast-like cells

We previously reported that PYK2 and p130 Cas were not tyrosine-phosphorylated in Src−/− OCLs (Lakkakorpi et al., 1999). Recently, tyrphostin A9 (or AG17), a tyrosine kinase inhibitor, was shown to be a potent inhibitor of TNF-induced PYK2 tyrosine phosphorylation in neutrophils (Fuortes et al., 1999). We therefore examined the effect of tyrphostin A9 and tyrphostin AG126, a structurally related tyrphostin with distinct inhibitory activity, on tyrosine-phosphorylated proteins in pOCs, following attachment to vitronectin-coated surfaces under serum-free conditions. As shown in Fig. 8A, tyrphostin A9 (1 and 10 μM) generally inhibited tyrosine phosphorylation of proteins in attached pOCs, while tyrphostin AG126 had minimal effects. We next examined adhesion-induced tyrosine phosphorylation of c-Src, PYK2 and paxillin in pOCs treated with these tyrphostins. Fig. 8B shows that 1 μM tyrphostin A9 partially, and 10 μM substantially, blocked tyrosine phosphorylation of c-Src, PYK2 and paxillin, while AG126 at the same concentrations had little effect. Interestingly, in in vitro kinase assays, which measure directly Src kinase activity, using enolase as substrate, or PYK2 activity estimated by autophosphorylation, tyrphostin A9 did not inhibit Src or PYK2 kinase activity at up to 10 μM concentration (data not shown). This suggests that another, as yet unknown, protein tyrosine kinase might be the direct target of tyrphostin A9, and responsible for blocking the adhesion-dependent activation of c-Src and PYK2 in osteoclasts.

Tyrphostin A9 induces hyperclustering of αvβ3 integrins and inhibits osteoclastic bone resorption

We next examined the effect of these tyrphostins on the morphology of wild-type osteoclasts. Tyrphostin A9 at 1 and 10 μM did not block attachment of pOCs to vitronectin, but inhibited cell spreading, whereas tyrphostin AG126 had no effect (Fig. 9A). Since tyrphostin A9 appeared to block the αvβ3 integrin-mediated signalling pathways in osteoclasts in a similar manner to that observed in Src-deficient cells, we investigated the effect of these tyrphostins on the formation of actin-rich rings in OCLs attached to tissue culture plates. Fig. 9B shows that tyrphostin A9 dose-dependently reduced the formation of actin-rich rings on plastic (IC50 approximately 1.0 μM). More importantly, tyrphostin A9 also inhibited bone resorption of OCLs (IC50 approximately 0.1 μM, Fig. 9C). In contrast, tyrphostin AG126 had no effect on bone resorption, even at 10 μM concentration (Fig. 9C), and had only a marginal effect on F-actin ring-like structure formation at high concentrations (Fig. 9B).

For immunolocalisation analysis of tyrphostin effects, OCLs were double stained for F-actin with paxillin, αvβ3, PYK2 or p130Cas in Src−/− and control OCLs were carried out as described in Materials and Methods. Confocal microscopy and conventional fluorescence microscopy were used to count cells in which these proteins colocalised at the bone surface. Data are expressed as percentage of cells showing colocalisation; numbers in parentheses indicate the numbers of Src−/− or control OCLs examined.

![Fig. 5. Localisation of PYK2 in control and Src-deficient OCLs on bone. Pseudocolored confocal microscopic images show localisation of PYK2 (a, in red), F-actin (c, in green) and their colocalisation as overlay images (c,f, in yellow). PYK2 localises to the sealing zone in wild-type cells (a-c), but very little PYK2 staining is seen in the intermediate sealing zone structures in Src−/− OCLs (d-f). Images merged from optical sections from 6.9 μm (a-c) and 1.6 μm (d-f) thickness close to the bone surface are shown. Bars, 10 μm.](image)

![Fig. 6. Colocalisation frequency of paxillin, αvβ3, PYK2 and p130Cas with F-actin in the ring-like structures in Src−/− and control OCLs on bone. Double stainings of F-actin with paxillin, αvβ3, PYK2 or p130Cas in Src−/− and control OCLs were carried out as described in Materials and Methods. Confocal microscopy and conventional fluorescence microscopy were used to count cells in which these proteins colocalised at the bone surface. Data are expressed as percentage of cells showing colocalisation; numbers in parentheses indicate the numbers of Src−/− or control OCLs examined.](image)
were first allowed to form sealing zones on bone prior to treatment with tyrosine kinase inhibitors. As seen in Fig. 10, numerous large clusters of $\alpha v \beta 3$ integrin-containing aggregates were found in the basal surface of OCLs treated with 0.1 $\mu$M tyrphostin A9 for 4 hours (e,f, in red), as compared to control OCLs (Fig. 10a,c, in red). Interestingly, tyrphostin A9-treated cells displayed actin rings that appeared to be less organised and significantly smaller than those in normal OCLs (compare a and d in Fig. 10). This may be an indication of impaired sealing zone formation, although 4 hours treatment may be too short to see the maximal effect. In OCLs treated with 1 $\mu$M tyrphostin AG126, aggregations of $\alpha v \beta 3$ integrins were occasionally observed as shown in Fig. 10h,i. However, the sealing zone structures in these cells were similar to those found in untreated OCLs (Fig. 10g). Taken together these observations suggest that tyrphostin A9 inhibited $\alpha v \beta 3$ integrin-signalling pathways in osteoclasts and induced a similar defective distribution of $\alpha v \beta 3$ integrins and of their downstream signalling and cytoskeletal molecules, as seen in Src-deficient osteoclasts.

**DISCUSSION**

Src-deficiency in mice is associated with osteopetrosis due to loss of osteoclast function, without a reduction in osteoclast number (Soriano et al., 1991). Morphologically, osteoclasts in Src$^{-/}$ mice are not able to form the resorptive apparatus, called ruffled border (Soriano et al., 1991; Boyce et al., 1992). In addition, Src-deficient osteoclasts do not resorb bone in vitro (Lowe et al., 1993). More recently, expression of wild-type or kinase-deficient c-Src in osteoclasts in Src$^{-/}$ mice was demonstrated to rescue the phenotype (Schwartzberg et al., 1997). Subsequent investigations documented the role of c-Src in osteoclast activation via phosphorylation of Cbl (Tanaka et al., 1996) and PYK2 (Duong et al., 1998). We reported that ligand engagement or clustering of $\alpha v \beta 3$ integrins in osteoclasts causes tyrosine phosphorylation and activation of the adhesion kinase PYK2 and the adapter protein p130Cas, and that c-Src is necessary for this pathway (Duong et al., 1993; Nakamura et al., 1998; Lakkakorpi et al., 1999). In the present study, we used Src-deficient osteoclasts to investigate the possible role of Src in $\alpha v \beta 3$ integrin mediated osteoclast actin ring (sealing zone) formation. We found that, although $\alpha v \beta 3$ integrin expression and ligand binding affinity are not changed in Src-deficient OCLs, defective $\alpha v \beta 3$ signalling seems to occur and interfere with normal localisation of this integrin as well as that of its downstream signalling molecules PYK2 and p130Cas and of the cytoskeletal proteins F-actin, vinculin and paxillin.
Osteoclasts appear to form sealing zones on bone surfaces in Src-deficient mice, as previously demonstrated by electron microscopic analysis (Boyce et al., 1992; Tanaka et al., 1992). We also found that Src−/− OCLs formed sealing zone-like structures on bone matrix in vitro, as documented by the distribution of microfilaments. However, the sealing zone-like structures found in these cells resemble incompletely formed ‘intermediate’ sealing zones, previously characterised in normal osteoclasts (Lakkakorpi and Väänänen, 1991). The organisation of microfilaments in these ‘intermediate’ sealing zones clearly differs from that in mature sealing zones of resorbing normal osteoclasts, which contain double circle structures of vinculin and paxillin. This observation suggests that the organisation of microfilaments during formation of the sealing zone in osteoclasts is impaired in the absence of c-Src. This is also consistent with previous studies showing disrupted F-actin organisation in Src-deficient osteoclasts on glass or plastic (Insogna et al., 1997; Duong et al., 1998).

The signalling proteins PYK2 and p130Cas, downstream of c-Src, were previously shown to localise to the sealing zone in osteoclasts during resorption (Duong et al., 1998; Lakkakorpi et al., 1999). Interestingly, these molecules were less frequently found in the intermediate sealing zones in Src−/− OCLs. This observation implicates Src-dependent tyrosine phosphorylation of PYK2 and p130Cas in the targeting of these molecules to the adhesion contacts of osteoclasts on bone. Previous studies using Src−/− cells have demonstrated that Src is required for integrin-induced cell spreading in fibroblasts (Kaplan et al., 1995; Felsenfeld et al., 1999; Klinghoffer et al., 1999) and CSF-1 induced spreading and reorganization of actin cytoskeleton in osteoclasts (Insogna et al., 1993). Although tyrosine phosphorylation of pp125FAK was not altered in Src−/− fibroblasts (Bockholt and Burridge, 1995), it was abolished in...
Src\(^{-/-}\)Yes\(^{-/-}\) Fyn\(^{-/-}\) triple mutant cells (Klinghoffer et al., 1999), suggesting compensation by other Src family kinases. On the other hand, p130\(^{Cas}\) tyrosine phosphorylation was virtually abolished in Src\(^{-/-}\) fibroblasts (Bockholt and Burridge, 1995) similar to the finding in Src\(^{-/-}\) osteoclasts (Lakkakorpi et al., 1999). Furthermore, similar to our finding of impaired localisation of p130\(^{Cas}\) to the sealing zone of Src\(^{-/-}\) osteoclasts, localisation of p130\(^{Cas}\) to focal adhesion contacts was abolished in Src\(^{-/-}\) fibroblasts (Nakamoto et al., 1997). Unlike fibroblasts, avian osteoclasts abundantly express c-Src, but very low levels of c-fyn, c-yes and c-lyn (Horne et al., 1992). However, murine osteoclasts express Hck and Fgr in addition to c-Src (Lowell et al., 1996). A severe form of osteopetrosis in Hck\(^{-/-}\)Src\(^{-/-}\) double mutants and increased Hck levels in Src\(^{-/-}\) osteoclasts suggest that Hck and Src may serve partially overlapping functions in osteoclasts (Lowell et al., 1996). Nevertheless, expression of other members of Src family kinases is not sufficient to rescue the bone resorption activity of Src-deficient osteoclasts. Recently, osteoclast function was shown to be inhibited by overexpression of Csk, a negative regulator of Src (Miyazaki et al., 2000), which resulted in decreased tyrosine phosphorylation of c-Cbl, PYK2 and p130\(^{Cas}\), and disrupted cytoskeletal organisation, resembling the observations in Src\(^{-/-}\) osteoclasts (Miyazaki et al., 2000).

An interesting finding in the present study was the hyperclustering of \(\alpha_\beta_3\) integrins and of their signalling partners PYK2 and p130\(^{Cas}\), as well as of microfilaments in Src-deficient osteoclasts on bone. This indicates that \(\alpha_\beta_3\) integrins form clusters and are able to associate with dephosphorylated PYK2 and p130\(^{Cas}\) and further recruit microfilaments to the membrane, suggesting that Src-mediated tyrosine phosphorylation might not be necessary for these processes. Indeed, we previously reported constitutive

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**Fig. 9.** Tyrphostin A9 inhibits cell spreading, actin ring formation and bone resorption in osteoclast-like cells. (A) pOCs were allowed to spread on vitronectin-coated dishes in the absence and presence of tyrphostins for 60 minutes, fixed and stained for TRAP activity. Phase-contrast images of untreated pOCs (a), or pOCs treated with 1 \(\mu\)M tyrphostin A9 (b) or 10 \(\mu\)M AG126 (c) are shown. Bar, 25 \(\mu\)m. (B) Similarly, OCLs were plated on tissue culture dishes without (C, 0 \(\mu\)M) and with tyrphostins (0.01-10 \(\mu\)M) for 4 hours. The percentages of TRAP(+) cells forming actin ring-like structures are shown at graded concentrations of tyrphostin A9 (open circles) or AG126 (closed circles). (C) OCLs were plated on bone for 4 hours, and then left untreated (cont.) or treated with indicated concentrations of tyrphostin A9 or AG126 for 20 hours. Pit areas were determined as described in Materials and Methods. Statistical differences, \(^*P<0.05\), \(^{**}P<0.01\), \(^{***}P<0.001\), from control are estimated with Kruskal-Wallis test; two-factor analysis of variance showed that A9 and AG126 are different from each other.

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association of PYK2 and p130Cas in osteoclasts in the absence of c-Src (Lakkakorpi et al., 1999). However, the hyperclustering observed in this study suggests that Src may be necessary for turnover and redistribution of the \( \alpha_v\beta_3 \) integrin-dependent signalling-microfilament complex to the sealing zone. The cause of hyperclustering at the basal surface, rather than at the membrane facing the bone surface, remains unclear, but it may reflect the dynamics of ligand engagement by \( \alpha_v\beta_3 \) integrins at the basal membrane. The significance of these aggregations needs further study.

In other systems, pp125FAK and Src were suggested to regulate the turnover of focal adhesions during cell migration. Fibroblast-like embryonic cells from FAK-deficient mice show reduced mobility and elevated number of more intensely stained focal adhesions in vitro (Ilic et al., 1995). Studies with v-Src have demonstrated that v-Src kinase activity and myristylation signals are required for disassembly of the v-Src/pp125FAK complex, degradation of pp125FAK and adhesion disruption during morphological transformation, but not for formation of focal adhesions (Fincham and Frame, 1998). On the other hand, it was shown that pp125FAK regulates activity and localisation of Src kinases, thus directing Src-mediated phosphorylation of focal adhesion-associated proteins (Schaller et al., 1999). In Src-deficient osteoclasts, we find reduced spreading and the aggregation of PYK2/p130Cas with microfilament proteins. It remains to be seen if hyperclustering of \( \alpha_v\beta_3 \) integrins and their associated proteins in Src-deficient osteoclasts reflects a similar phenomenon to changes in focal adhesion turnover caused by impaired pp125FAK/Src signalling.

Recent studies report interesting data on binding forces of integrin-ligand using atomic force microscopy (Lehenkari and Horton, 1999) and of integrin-cytoskeleton interactions, using laser trap of ligand-coated beads (Felsenfeld et al., 1999). Felsenfeld et al. showed that Src selectively promotes the reversibility of vitronectin receptor-cytoskeleton interactions needed for cell spreading and migration, without changing ligand binding (Felsenfeld et al., 1999). It is therefore tempting to speculate that hyperclustering of \( \alpha_v\beta_3 \) integrins and their associated proteins in Src-deficient osteoclasts represents tight or ‘frozen’ integrin-cytoskeleton interactions, causing impaired osteoclast spreading, migration and function.

In addition to the genetic approach, which focuses on c-Src, we also used in this study a pharmacological approach to characterise the involvement of tyrosine kinases in \( \alpha_v\beta_3 \)-mediated osteoclast activation and function. Recently, the tyrosine kinase inhibitor tyrphostin A9 has emerged as a potent inhibitor of TNF-induced PYK2 tyrosine phosphorylation in
neutrophils (Fuortes et al., 1999). In addition, tyrphostin A9 inhibited Ca\textsuperscript{2+} entry triggered by depletion of Ca\textsuperscript{2+} stores in T lymphocytes, which was independent of T cell receptor engagement (Marhaba et al., 1996). Here, we find that tyrphostin A9, but not the structurally related but functionally distinct tyrphostin AG126, inhibited adhesion-induced tyrosine phosphorylation of c-Src, PYK2 and Paxillin in osteoclasts. However, in vitro kinase assays failed to demonstrated a direct effect of tyrphostin A9 on Src or PYK2 kinase activity, indicating that tyrphostin A9 has a different in vivo target in osteoclasts, which remains to be investigated. The possibility for the involvement of additional kinases was suggested when Src\textsuperscript{\textminus/} mice were rescued with kinase-deficient Src (Schwartzberg et al., 1997). The importance of tyrosine kinases in osteoclast function is suggested by the pronounced effects of tyrphostin A9 on inhibition of cell spreading, reduction of actin ring formation on plastic, inhibition of bone resorption and induction of hyperclustering of $\alpha$3$\beta$3 integrins in osteoclasts. Treatment with tyrphostin A9 resulted in very similar changes to those seen in Src-deficient osteoclasts, suggesting that tyrphostin A9 blocks the $\alpha$3$\beta$3 integrin-dependent signalling pathway via c-Src and PYK2, thus inhibiting osteoclast activation and function. Tyrphostin A9 could be used together with Src-deficient cells to dissect upstream as well as downstream targets of PYK2 and c-Src in osteoclasts.

In summary, the data presented here show that although $\alpha$3$\beta$3 integrin expression and ligand binding affinity are not altered in Src-deficient osteoclasts, lack of Src-dependent $\alpha$3$\beta$3-mediated signals seems to interfere with normal turnover of $\alpha$3$\beta$3-associated protein complexes, resulting in abnormal targeting and localisation of the integrins and of their downstream effectors. This suggests a crucial role for Src in the dynamic interactions between $\alpha$3$\beta$3 integrins and their associated proteins, which are important for complete osteoclast polarisation during the resorption process. Moreover, treatment with the tyrosine kinase inhibitor tyrphostin A9, although its direct target is unknown, results in similar morphological and cellular changes, as seen in Src-deficient osteoclasts. Taken together, these data raise the possibility that in the adhesion-dependent signalling pathway in osteoclasts, $\alpha$3$\beta$3 integrin-mediated recruitment of downstream effectors does not require Src-dependent tyrosine phosphorylation of these molecules. Src kinase activity might be important for the turnover of the integrin-associated complex.

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