Convergence of $\alpha_v\beta_3$ Integrin– and Macrophage Colony Stimulating Factor–mediated Signals on Phospholipase Cγ in Prefusion Osteoclasts

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Abstract. The macrophage colony stimulating factor (M-CSF) and $\alpha_v\beta_3$ integrins play critical roles in osteoclast function. This study examines M-CSF– and adhesion-induced signaling in prefusion osteoclasts (pOCs) derived from Src-deficient and wild-type mice. Src-deficient cells attach to but do not spread on vitronectin (Vn)-coated surfaces and, contrary to wild-type cells, their adhesion does not lead to tyrosine phosphorylation of molecules activated by adhesion, including PYK2, p130^{Cas}, paxillin, and PLC-γ. However, in response to M-CSF, Src$^{-/-}$ pOCs spread and migrate on Vn in an $\alpha_v\beta_3$-dependent manner. Involvement of PLC-γ activation is suggested by using a PLC inhibitor, U73122, which blocks both adhesion- and M-CSF–mediated cell spreading. Furthermore, in Src$^{-/-}$ pOCs M-CSF, together with filamentous actin, causes recruit-

ment of β_3 integrin and PLC-γ to adhesion contacts and induces stable association of β_3 integrin with PLC-γ, phosphatidylinositol 3-kinase, and PYK2. Moreover, direct interaction of PYK2 and PLC-γ can be induced by either adhesion or M-CSF, suggesting that this interaction may enable the formation of integrin-associated complexes. Furthermore, this study suggests that in pOCs PLC-γ is a common downstream mediator for adhesion and growth factor signals. M-CSF–initiated signaling modulates the $\alpha_v\beta_3$ integrin-mediated cytoskeletal reorganization in prefusion osteoclasts in the absence of c-Src, possibly via PLC-γ.

Key words: $\alpha_v\beta_3$ integrins • osteoclasts • M-CSF • Src kinases • phospholipase Cγ

Introduction

Integrins are transmembrane heterodimeric glycoproteins consisting of α and β subunits that mediate cell–cell and cell–matrix interactions. Ligand binding to integrins activates signal transduction pathways which lead to de novo gene expression and cytoskeletal rearrangement associated with cell adhesion, spreading, and migration (Thomas and Brugge, 1997; Giancotti and Ruoslahti, 1999). It has been shown that integrins activate multiple signaling pathways including elevation of intracellular Ca^{2+}, lipid turnover, and tyrosine phosphorylation. The proteins which are tyrosine phosphorylated by extracellular matrix (ECM)[1]–integrin interactions include the focal adhesion kinases (FAKs) or PYK2/CAKβ/RAFTK/CADTK, in certain cell types, p130^{Cas}, and cytoskeletal molecules such as

paxillin, tensin, and cortactin (Thomas and Brugge, 1997; Giancotti and Ruoslahti, 1999; Schlaepfer et al., 1999).

Several lines of evidence indicate that integrin-mediated signals synergize with growth factor responses to produce the structural changes associated with cell migration, proliferation, and differentiation (Sastry and Horwitz, 1996; Giancotti and Ruoslahti, 1999; Sieg et al., 2000). First, many signaling molecules found in integrin-dependent focal adhesions, such as Src or phosphatidylinositol 3-kinase (PI 3-kinase), are also known to associate with tyrosine kinase growth factor receptors (Schwartz and Ingber, 1994; Yamada and Miyamoto, 1995). Second, adhesion of most nontransformed cells to ECM is required for cellular responses to growth factor stimulation and, in some instances, directly regulates growth factor expression (Soldi et al., 1999). Third, growth factors and integrins often reciprocally regulate cellular responses such as cell migration (Plopper et al., 1995; Filardo et al., 1996; Sieg et al., 2000).

Osteoclasts are macrophage-related multinucleated cells responsible for the degradation of mineralized matrix (Suda et al., 1996). Their adhesion to the bone surface induces the cytoskeletal reorganization associated with activation, suggesting that recognition of bone ECM proteins is an important step in the initiation of osteoclastic bone resorption (Duong and Rodan, 1998). Although osteoclasts express

$\alpha_2\beta_1$ and $\alpha_v\beta_1$ integrins, their predominant integrin is $\alpha_v\beta_3$. Disintegrins, $\alpha_v\beta_3$ blocking antibodies, and RGD peptide mimetics have been shown to inhibit bone resorption in vitro and in vivo. We reported that PYK2 and p130^{Cas} are key effectors in the $\alpha_v\beta_3$ integrin–mediated signaling pathways, and their activation requires c-Src in osteoclasts (Duong et al., 1998; Lakkakorpi et al., 1999). Osteoclasts are also target cells for several cytokines and growth factors, among which macrophage colony stimulating factor (M-CSF) is essential for both osteoclast development and function (Felix et al., 1994). A role for M-CSF in osteoclast formation was first identified in the osteopetrotic *op/op* mice. Subsequent reports showed that mature osteoclasts also contain the M-CSF receptor, *c-fms*, which is required for the survival, spreading, and migration of these cells.

The object of this study was to investigate interactions between $\alpha_v\beta_3$ integrin–mediated and M-CSF–dependent signaling pathways in osteoclasts. We found that Src-deficient prefusion osteoclasts (pOCs) adhered to, but failed to spread on vitronectin (Vn)-coated surfaces. $\alpha_v\beta_3$ integrin–mediated signaling was abolished in these cells since several adhesion-dependent molecules including PYK2, p130^{Cas}, PLC-γ, and paxillin were not tyrosine phosphorylated upon attachment to Vn. However, M-CSF induced cell spreading of Src-deficient pOCs in an integrin-dependent manner, and an inhibitor of PLC-γ blocked the M-CSF–dependent cell spreading. In addition, we found that in Src-deficient cells, M-CSF initiated the recruitment of $\alpha_v\beta_3$ integrin and PLC-γ to adhesion contacts. M-CSF also induced the association of $\alpha_v\beta_3$ integrin with several signaling molecules including PLC-γ, PI 3-kinase, and PYK2 in a Src-independent manner, which was blocked by a PLC inhibitor. The interaction between $\alpha_v\beta_3$ and these molecules in pOCs seems to depend on the association of PYK2 and PLC-γ. These data suggest that PLC-γ is an important effector of $\alpha_v\beta_3$- and M-CSF–mediated signaling pathways involved in prefusion osteoclast spreading.

Materials and Methods

Antibodies and Other Reagents

Vn and poly-L-lysine (PL) were from GIBCO BRL and Sigma-Aldrich, respectively. Antibodies to PYK2 (N-19), PLC-$\gamma1$ (1249 and mAb E-12), PLC-$\gamma2$ (Q20 and mAb B-10), phospho–extracellular signal–regulated kinase (ERK) (E-4), and ERK2 (C-14) were from Santa Cruz Biotechnology, Inc. Antibodies to p130^{Cas} (mAb 21), paxillin (mAb 349), PYK2 (mAb 11), and phosphotyrosine (mAb PY20) were from Transduction Labs. Anti–β_3 integrin antibodies (mAb 2C9.G2) were from BD PharMingen. Anti-Akt/PKB and anti–phospho-Akt/PKB antibodies were from New England Biolabs, Inc. Anti–phosphotyrosine antibody (mAb 4G10) was from Upstate Biotechnology. Other conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories and Amersham Pharmacia Biotech. Glutathione *S*-transferase (GST) fusion proteins of PLC-$\gamma1$ were from Santa Cruz Biotechnology, Inc. Collagenase was from Wako Chemicals and dispase from Boehringer. $1\alpha,25$-dihydroxyvitamin D$_3$ ($1\alpha,25[OH]_2D_3$) was a gift from Dr. M. Uskokovic (Hoffmann-LaRoche, Nutley, NJ). Mouse recombinant M-CSF was from R&D Systems. Wortmannin, LY294002, U73122, and PD98059 were purchased from Calbiochem. Echistatin and polyclonal anti-$\beta3$ integrin antibodies were generously provided by Drs. W.K. Herber and B. Bednar (Merck Research Laboratories, West Point, PA).

Animals

Balb/C mice were obtained from Taconic Farms. Heterozygote Src$^{+/-}$ mice were obtained from The Jackson Laboratory and Src$^{-/-}$ mice were phenotypically distinguished from their Src$^{+/?}$ siblings by lack of tooth eruption. All animals were cared and housed under conditions approved by the Institutional Animal Care and Use Committee Guide.

Cell Cultures

Prefusion osteoclast-like cells (pOCs) and multinucleated osteoclast-like cells (OCLs) were prepared as described previously with slight modifications (Duong et al., 1998). In brief, spleen cells isolated from 2–3-wk-old Src$^{-/-}$ or their normal littermates were cocultured with osteoblastic MB1.8 cells for 5–6 d in the presence of 10 nM $1\alpha,25(OH)_2D_3$. pOCs were released from dishes with 10 mM EDTA after removing MB1.8 cells with collagenase-dispase. Alternatively, cocultures were kept for 7–8 d to achieve OCLs and purified as described previously (Duong et al., 1998).

Cell Adhesion

After isolation, pOCs (3×10^5 cells/condition) were washed twice with serum-free α-MEM containing 0.1% BSA (Sigma-Aldrich) and kept in suspension or allowed to attach to polystyrene dishes coated with Vn (20 μg/ml) or PL (50 μg/ml). After 5–60 min at 37°C, an equal volume of 2× TNE lysis buffer (20 mM Tris, pH 7.8, 300 mM NaCl, 2 mM EDTA, 2% NP-40, 2 mM NaVO$_3$, 20 mM NaF, 20 μg/ml leupeptin, 1 TIU/ml aprotinin, and 2 mM PMSF) was added to the plates. For coimmunoprecipitation, 1.5×10^6 cells/condition and 1× TNE lysis buffer with 10% glycerol (10 nM Tris, pH 7.8, 300 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM NaVO$_3$, 10 mM NaF, 10 μg/ml leupeptin, 0.5 TIU/ml aprotinin, and 1 mM PMSF) were used. In some experiments, pOCs were recultured for 12 h with $1\alpha.25(OH)_2D_3$-pretreated osteoblastic MB1.8 cells to form multinucleated OCLs, which were subsequently purified by removing MB1.8 cells using collagenase/dispase, as described. Clarified lysates were subjected to immunoprecipitation and immunoblotting. Alternatively, cells were fixed and stained for tartrate resistant acid phosphatase (TRAP), a marker enzyme of osteoclasts (Nakamura et al., 1999). To quantify cell area, the periphery of each cell was outlined and the total planar area was calculated using an image analysis system (Empire Imaging Analyzing Systems).

Immunoblotting and Immunoprecipitation

Immunoprecipitation and immunoblotting were performed as described previously (Duong et al., 1998). In brief, lysates were precipitated with anti-PYK2, p130^{Cas}, paxillin, PLC-$\gamma1$, PLC-$\gamma2$, or integrin β_3 antibodies (2 μg) for 2 h at 4°C, followed by protein G–Sepharose for 1 h at 4°C. After washing four times with lysis buffer, proteins were separated on an 8% SDS-PAGE and blotted onto Immobilon-P membrane. After blocking with 100 mM NaCl, 10 mM Tris, 0.1% Tween-20, and 2% BSA, the membrane was incubated with primary antibodies, followed by HRP-conjugated secondary antibodies and detected with the ECL chemiluminescence system (Amersham Pharmacia Biotech). Levels of proteins in immunoblots were quantitated using an Imaging Densitometer (model GS-700, BioRad) and specific activity of tyrosine phosphorylated proteins at various time points were estimated from the ratio of phosphorylated proteins to its protein content, and expressed relative to controls at time 0.

In Vitro Protein Association Assays

These experiments were performed with GST fusion proteins containing the Src homology (SH) 3 domain, SH2 domains, both SH2 domains, and SH3 domain of PLC-$\gamma1$. Multinucleated osteoclast-like cell lysates (1 mg/ml) were incubated with GST fusion protein coupled with glutathione-Sepharose beads for 2 h at 4°C. The beads were washed three times with lysis buffer and one time with PBS, and precipitated proteins were separated by SDS-PAGE and subjected to immunoblot analysis using anti-PYK2 antibodies as described above.

Immunofluorescence

Src-deficient pOCs were cultured for 1 h on glass coverslips. After cells were treated with 5 nM M-CSF for another 30 min, cells were fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100 in PBS, and incubated for 30 min at 37°C with polyclonal anti–β_3 integrin (Nakamura et al., 1999) and monoclonal anti–PLC-$\gamma1$ or anti–PLC-$\gamma2$ antibodies. Cells were washed with PBS and incubated for 30 min at 37°C with TRITC-conjugated donkey anti–rabbit IgG and FITC goat anti–mouse IgG. Samples were viewed with a Leica TCS SP Spectral confocal laser scanning microscope equipped with Argon-Krypton laser (Leica Microsystems).
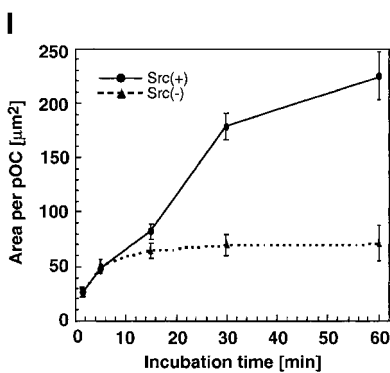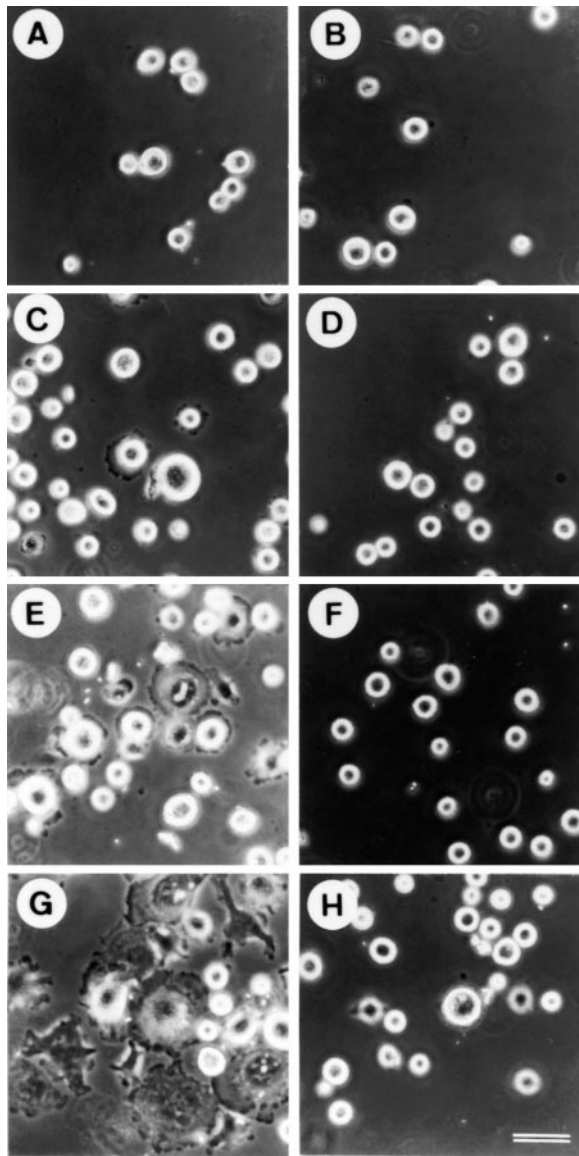
Figure 1. Src$^{-/-}$ pOCs do not spread on Vn-coated dishes. Src$^{+/?}$ (A, C, E, and G) and Src$^{-/-}$ (B, D, F, and H) pOCs were plated on Vn (20 μg/ml). After culture for 5 (A and B), 15 (C and D), 30 (E and F), and 60 (G and H) min, cells were fixed and photographed. (I) To quantify cell area, the periphery of each cell was outlined and the total planar area was calculated, using an image analysis system (Empire Imaging Systems). Data are expressed as the means of ± SEM of >50 cells.

Cell Migration

Migration assay was performed as described by Nakamura et al. (1999), in which Src$^{+/?}$ or Src$^{-/-}$ pOCs were cultured on Vn-coated dishes in medium 199 (25 mM Hepes, 4 mM HCO$_3^-$, supplemented with 0.1% FBS), covered with paraffin oil, and maintained at 35°C using a stage heater. Osteoclasts were observed using an inverted phase–contrast microscope coupled to a video camera and time-lapse video recorder (one frame per 2 s). Using M-CSF as a chemotactic stimulus, osteoclast migration was monitored. A micropipette containing M-CSF (1 nM) was positioned 200–400 μm from the cells and contents were delivered by a syringe pump at the rate of 4 μl/h (Harvard Apparatus). Osteoclast responses were recorded for 4 h by time-lapse video microscopy. Images of pOCs were digitized using an analysis system (Empire Imaging Systems) and migration was quantified as the net movement of the cell centroid during a culture period of 4 h.

Results

M-CSF Induces Cell Spreading and Migration of Src$^{-/-}$ Osteoclasts in an $\alpha_v\beta_3$ Integrin–dependent Manner

Fibroblasts from Src-deficient mice were shown to have a reduced rate of spreading on fibronectin (Kaplan et al., 1995). We found that the pOCs derived from Src$^{-/-}$ mice exhibit a profound defect in cell spreading on Vn-coated surfaces (Fig. 1). Wild-type pOCs fully spread within 60 min of plating (Fig. 1, left), whereas Src$^{-/-}$ pOCs remained rounded at 60 min (Fig. 1, right), and up to 120 min (data not shown). Spreading area of wild-type and Src-deficient pOC on Vn were quantitated and are shown in Fig. 1 I. Initial attachment to Vn appeared to be normal in Src$^{-/-}$ pOCs; however, these cells were easily detached by shaking and tapping, indicating that the firm adhesion associated with cell spreading did not occur, although the expression level of $\alpha_v\beta_3$ integrins and their binding affinity were not altered in Src$^{-/-}$ pOCs (Lakkakorpi et al., 2000). The highest number of Src$^{-/-}$ pOCs attached to Vn-coated dishes was observed 60 min after seeding.

It has been shown that the M-CSF receptor, c-Fms, is expressed in mature osteoclasts and that M-CSF induces cell spreading and cell migration in rat primary osteoclasts and murine osteoclast-like cells (Felix et al., 1994). To determine whether c-Src function is required for M-CSF–induced cytoskeletal reorganization during cell spreading and migration, Src-deficient and wild-type pOCs were plated on Vn-coated dishes and treated with M-CSF. To obtain optimal numbers of attached cells, we first allowed Src$^{-/-}$ cells to adhere to Vn-coated surfaces for 60 min prior to M-CSF addition. Although Src$^{-/-}$ pOCs did not spread spontaneously on Vn, M-CSF rapidly induced Src$^{-/-}$ cell spreading (Figs. 2 A and 3, first and second bars). Moreover, M-CSF induced the formation of small punctate adhesion contacts in Src$^{-/-}$ pOCs, similar to podosomal adhesion structures found in wild-type cells (Fig. 3 B). Because a previous study found that 2.5 nM M-CSF did not induce cell spreading of nonpurified primary Src-deficient osteoclasts (Insogna et al., 1997), we examined cell spreading of wild-type and Src$^{-/-}$ pOCs at 0, 2.5 and 5.0 nM M-CSF ($n = 50$), to rule out a dose effect phenomenon. The cell area of untreated wild-type cells was 234 ± 41 μm^2 and of Src$^{-/-}$ pOCs, 93 ± 15 μm^2. M-CSF at 2.5 nM increased the cell area in wild-type to 279 ± 16 μm^2 (119%) and in Src$^{-/-}$ pOC to 203 ± 22 μm^2 (218%), respectively; while 5 nM M-CSF increased cell spreading area to 318 ± 25 μm^2
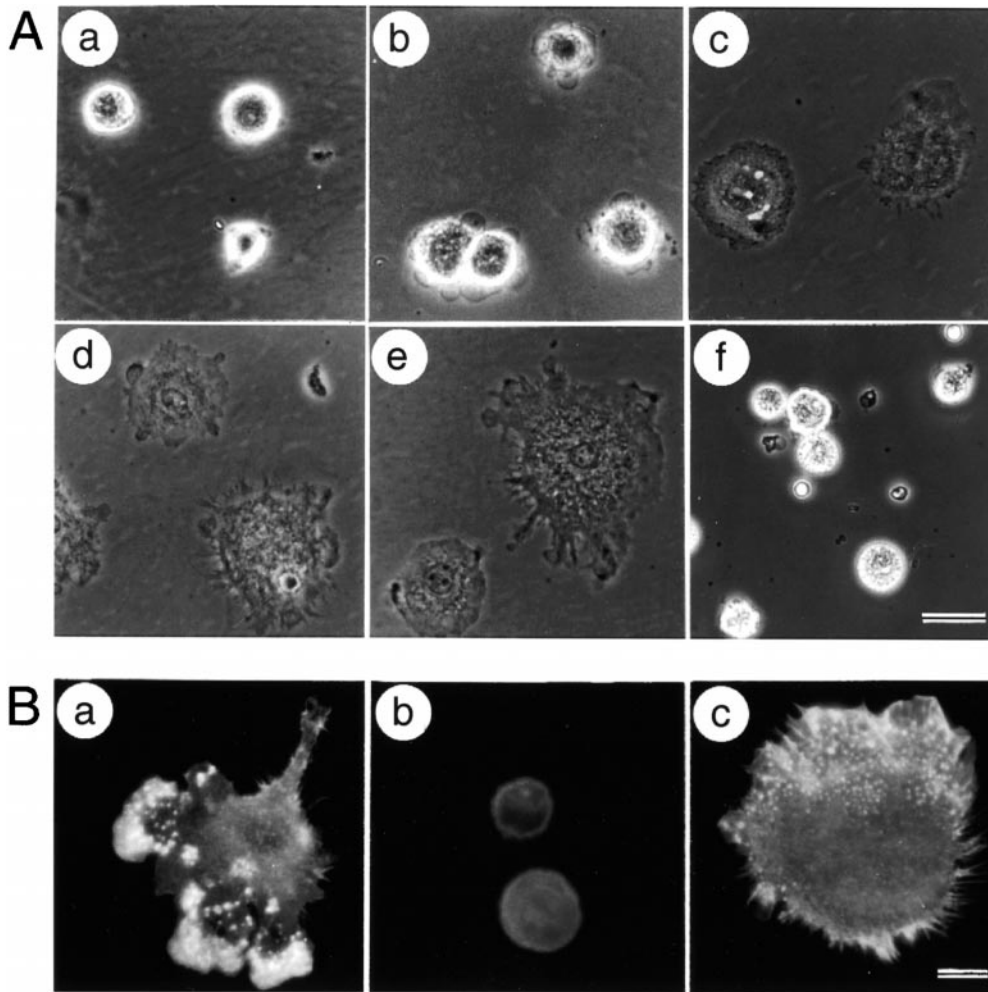
Figure 2. M-CSF induces cell spreading of Src$^{-/-}$ pOCs on Vn-coated dishes. (A) Src$^{-/-}$ pOCs were plated on Vn-coated dishes in the absence of serum for 60 min, cells were then treated with 5 nM M-CSF for 0 (a), 2 (b), 5 (c), 15 (d), and 30 (e) min, without or with (f) 100 nM wortmannin. (B) Src$^{+/?}$ (a) and Src$^{-/-}$ (b and c) pOCs were plated on Vn for 60 min, untreated (a and b) or treated with 5 nM M-CSF treatment for additional 30 min (c). Cells were fixed and stained with rhodamine-conjugated phalloidin. Bars: (A) 10 μm; (B) 5 μm.

(135%) in wild-type and 270 ± 27 μm² (290%) in Src$^{-/-}$ pOC, respectively; i.e. at both doses, there was a pronounced effect on the spreading of Src$^{-/-}$ pOCs, and not of wild-type pOCs.

Furthermore, M-CSF–stimulated osteoclast chemotaxis of Src-deficient cells was not different from wild-type cells (Fig. 4). In control cultures 15 out of 26 (58%) Src$^{+/?}$ pOCs migrated towards the source of M-CSF, and the net migration distance over a 4-h period was 25.5 ± 2.0 μm (means ± SEM). Similarly, 10 out of 19 (53%) Src$^{-/-}$ pOCs showed chemotactic migration, and the net distance was 24.5 ± 2.9 μm, not significantly different from wild-type. These observations suggest that Src function is not required for the M-CSF–induced cytoskeletal reorganization required for osteoclast spreading and migration.

To further examine the role of $\alpha_v\beta_3$ integrin in M-CSF–induced Src$^{-/-}$ pOC spreading, cells were plated on Vn- or PL-coated dishes in the presence of M-CSF under serum-free conditions. As shown in Fig. 3, M-CSF–induced cell spreading of Src$^{-/-}$ pOCs only when cells were plated on Vn, but not on PL. It should be noted that wild-type pOCs plated on PL do not spread either in the absence or presence of M-CSF (data not shown). Moreover, M-CSF–induced Src-deficient pOC cell spreading on Vn was blocked by the RGD-containing disintegrin, echistatin (Fig. 3) which was previously demonstrated to have high binding affinity for $\alpha_v\beta_3$ and to inhibit $\alpha_v\beta_3$-mediated spreading, migration, and sealing zone formation in osteoclasts (Nakamura et al., 1999). This finding suggested that in the absence of c-Src, M-CSF-initiated cytoskeletal rearrangement to during cell spreading and migration still depends

ECM	VN	VN	PL	VN
M-CSF (5 nM)	-	+	+	+
Echistatin (1 nM)	-	-	-	+
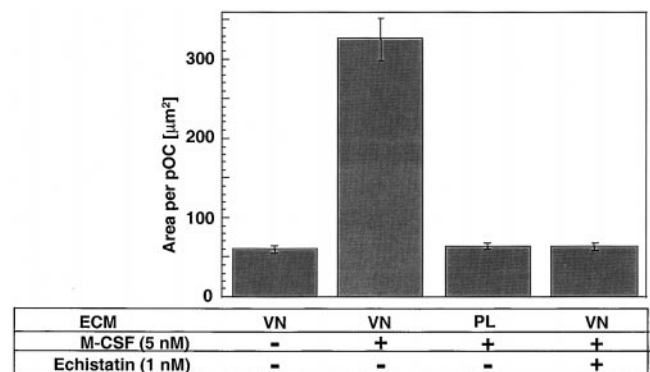
Figure 3. M-CSF–induced cell spreading of Src$^{-/-}$ pOCs is dependent on $\alpha_v\beta_3$ integrin. Src$^{-/-}$ pOCs were plated on Vn or PL in serum-free condition. After 60 min, cells were treated with 5 nM M-CSF for 30 min in the absence or presence of echistatin (1 nM). Cells were fixed and stained for TRAP activity, followed by quantitating cell area as described above. Data are presented as means ± SEM and n = 50 cells per group.
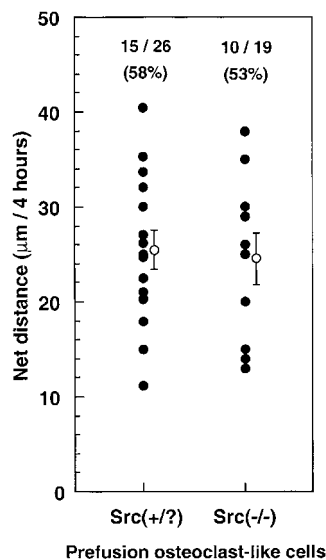
Figure 4. M-CSF induces cell migration of Src$^{-/-}$ pOCs as well as wild-type cells. The motility of Src$^{+/?}$ and Src$^{-/-}$ pOCs was monitored using time-lapse video microscopy, as described in Materials and Methods. M-CSF (1 nM) was supplied through a micropipette to induce chemotaxis. Cells were observed using an inverted phase–contrast microscope coupled to a video camera and time-lapse video recorder. Images of cells were digitized using a computer-based image analysis system. Migration activity (net translocation of the cell center over a period of 4 h) was quantified (filled circles). 15 out of 26 (58%) Src$^{+/?}$ cells and 10 out of 19 (53%) Src$^{-/-}$ cells migrated towards the source of M-CSF. Means ± SEM (open circles) of migrating distance of wild-type and Src$^{-/-}$ cells are shown.

on ligand engagement of $\alpha_v\beta_3$ integrins. We had previously shown that tyrosine phosphorylation of PYK2 and p130^{Cas}, $\alpha_v\beta_3$-associated downstream signaling molecules, is significantly diminished in Src-deficient OCLs (Duong et al., 1998; Lakkakorpi et al., 1999). We therefore searched for potential downstream participants, which could modulate the synergistic action of the adhesion- and M-CSF-mediated cytoskeletal reorganization in Src-deficient pOCs and evaluated include PI 3-kinase and PLC.

Inhibitors of PLC and PI 3-Kinase Block Adhesion- and M-CSF–induced Cell Spreading of Src-deficient Osteoclasts

We examined the involvement of PI 3-kinase and PLC-γ in M-CSF–mediated signaling in Src-deficient cells using either wortmannin (0.1 μM) or U73122 (1 μM). As shown in Fig. 5 A (bars 5–7) and Fig. 2 F, either inhibitor blocked M-CSF–induced cell spreading in Src$^{-/-}$ pOCs. Both inhibitors also blocked the migration of wild-type and Src-deficient cells assessed by time-lapse video microscopy (data not shown). Similarly, cell spreading of wild-type pOCs upon adhering to Vn was also inhibited by wortmannin (data not shown) and U73122 (Fig. 5 A, bars 1 and 2). Although U73122 is widely used as a PLC inhibitor, it has been shown to interfere with non PLC-dependent signals, usually at higher concentrations (>10 μM) (Walker et al., 1998). Nevertheless, our pharmacological findings suggest that PI 3-kinase and PLC-γ may take part in both adhesion-dependent and M-CSF–mediated signaling, which leads to cytoskeletal organization in prefusion osteoclasts.

Interestingly, PD98059, a mitogen-activated protein (MAP) kinase kinase inhibitor, had little effect on the spreading of Src$^{-/-}$ pOCs (Fig. 5 A, bars 5 and 8), although activation of ERK1 and 2 were induced by attachment to Vn-coated surface and by M-CSF treatment in wild-type pOCs using phospho-ERK–specific antibodies (Fig. 5 B, right). However, these kinases were not activated by either
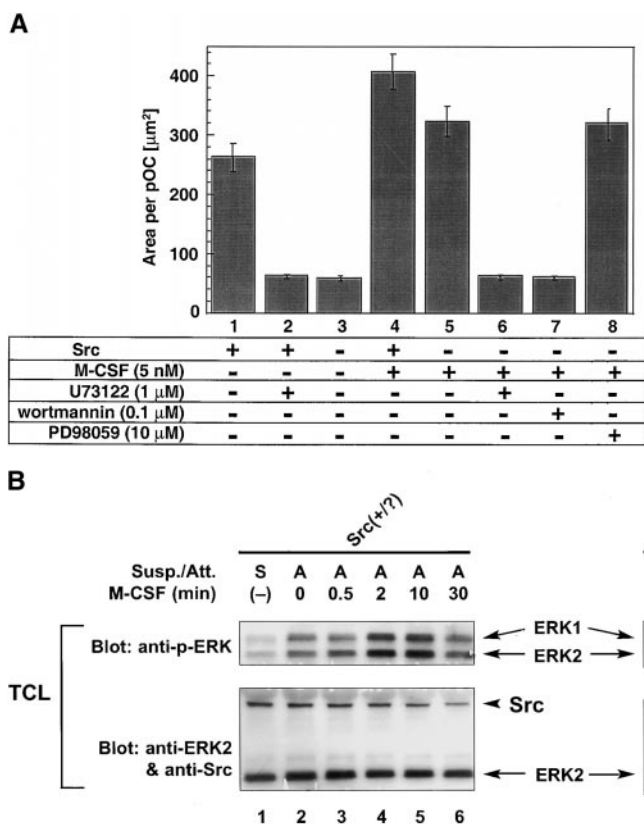

A

Src	+	+	-	+	-	-	-	-
M-CSF (5 nM)	-	-	-	+	+	+	+	+
U73122 (1 μM)	-	+	-	-	-	+	-	-
wortmannin (0.1 μM)	-	-	-	-	-	-	+	-
PD98059 (10 μM)	-	-	-	-	-	-	-	+

B

Susp./Att.	S	A	A	A	A	A
M-CSF (min)	(–)	0	0.5	2	10	30

Susp./Att.	S	A	A	A	A	A	A
M-CSF (min)	(–)	0	0.5	2	10	30	30

Figure 5. PLC and PI 3-kinase inhibitors block adhesion-induced and M-CSF–induced cell spreading of prefusion osteoclast-like cells. (A) Src$^{+/?}$ pOCs (1, 2, and 4) and Src$^{-/-}$ pOCs (3 and 5–8) were plated on Vn under serum-free condition. After 60 min, cells were treated with (4–8) or without (1–3) M-CSF for 30 min. U73122 (2 and 6), wortmannin (7), or PD98059 (8) was preincubated for 40 min before M-CSF was added. Cells were fixed, stained for TRAP, and the total planar area was calculated as described in Materials and Methods. Data are presented as means ± SEM; $n = 50$ cells per group. (B) Src$^{+/?}$ and Src$^{-/-}$ pOCs were kept in suspension or plated on Vn for 60 min in the absence of serum, followed by the treatment with 5 nM M-CSF for indicated periods with or without 10 μM PD98059. Lysates were blotted with anti–phospho-ERK (top), followed by anti-ERK and anti-Src (bottom). Susp., suspension; Att., attachment.
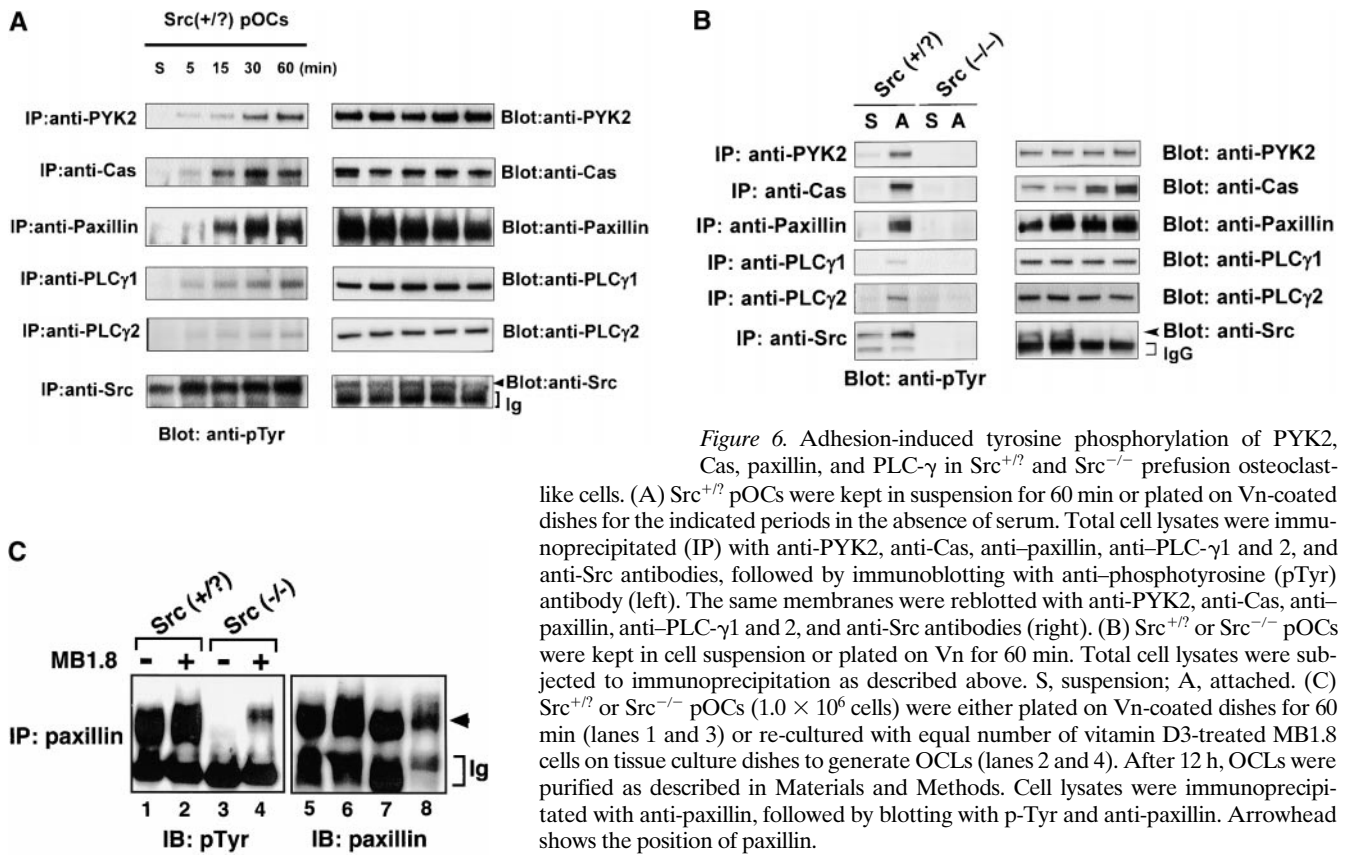
A Src(+/?) pOCs

S 5 15 30 60 (min)

IP:anti-PYK2 / Blot:anti-PYK2
IP:anti-Cas / Blot:anti-Cas
IP:anti-Paxillin / Blot:anti-Paxillin
IP:anti-PLCγ1 / Blot:anti-PLCγ1
IP:anti-PLCγ2 / Blot:anti-PLCγ2
IP:anti-Src / ◄Blot:anti-Src] Ig

Blot: anti-pTyr

B

Src(+/?) Src(−/−)
S A S A

IP: anti-PYK2 / Blot: anti-PYK2
IP: anti-Cas / Blot: anti-Cas
IP: anti-Paxillin / Blot: anti-Paxillin
IP: anti-PLCγ1 / Blot: anti-PLCγ1
IP: anti-PLCγ2 / Blot: anti-PLCγ2
IP: anti-Src / ◄Blot: anti-Src] IgG

Blot: anti-pTyr

C

Src(+/?) Src(−/−)
MB1.8 − + − +

IP: paxillin

1 2 3 4   5 6 7 8
IB: pTyr   IB: paxillin

Figure 6. Adhesion-induced tyrosine phosphorylation of PYK2, Cas, paxillin, and PLC-γ in Src$^{+/?}$ and Src$^{-/-}$ prefusion osteoclast-like cells. (A) Src$^{+/?}$ pOCs were kept in suspension for 60 min or plated on Vn-coated dishes for the indicated periods in the absence of serum. Total cell lysates were immunoprecipitated (IP) with anti-PYK2, anti-Cas, anti–paxillin, anti–PLC-γ1 and 2, and anti-Src antibodies, followed by immunoblotting with anti–phosphotyrosine (pTyr) antibody (left). The same membranes were reblotted with anti-PYK2, anti-Cas, anti–paxillin, anti–PLC-γ1 and 2, and anti-Src antibodies (right). (B) Src$^{+/?}$ or Src$^{-/-}$ pOCs were kept in cell suspension or plated on Vn for 60 min. Total cell lysates were subjected to immunoprecipitation as described above. S, suspension; A, attached. (C) Src$^{+/?}$ or Src$^{-/-}$ pOCs (1.0 × 10^6 cells) were either plated on Vn-coated dishes for 60 min (lanes 1 and 3) or re-cultured with equal number of vitamin D3-treated MB1.8 cells on tissue culture dishes to generate OCLs (lanes 2 and 4). After 12 h, OCLs were purified as described in Materials and Methods. Cell lysates were immunoprecipitated with anti-paxillin, followed by blotting with p-Tyr and anti-paxillin. Arrowhead shows the position of paxillin.

pathway in Src$^{-/-}$ pOCs (Fig. 5 B, left). These findings suggest that adhesion- and M-CSF–dependent activation of the MAP kinases require c-Src in pOCs. However, in Src-deficient prefusion osteoclasts their activation did not correlate with M-CSF–induced cytoskeletal rearrangement.

Adhesion-mediated Protein Tyrosine Phosphorylation Is Impaired in Src-deficient Osteoclasts

Initial events triggered by integrin engagement of ECM ligands include recruitment and phosphorylation of numerous signaling and cytoskeletal molecules, leading to cyto-
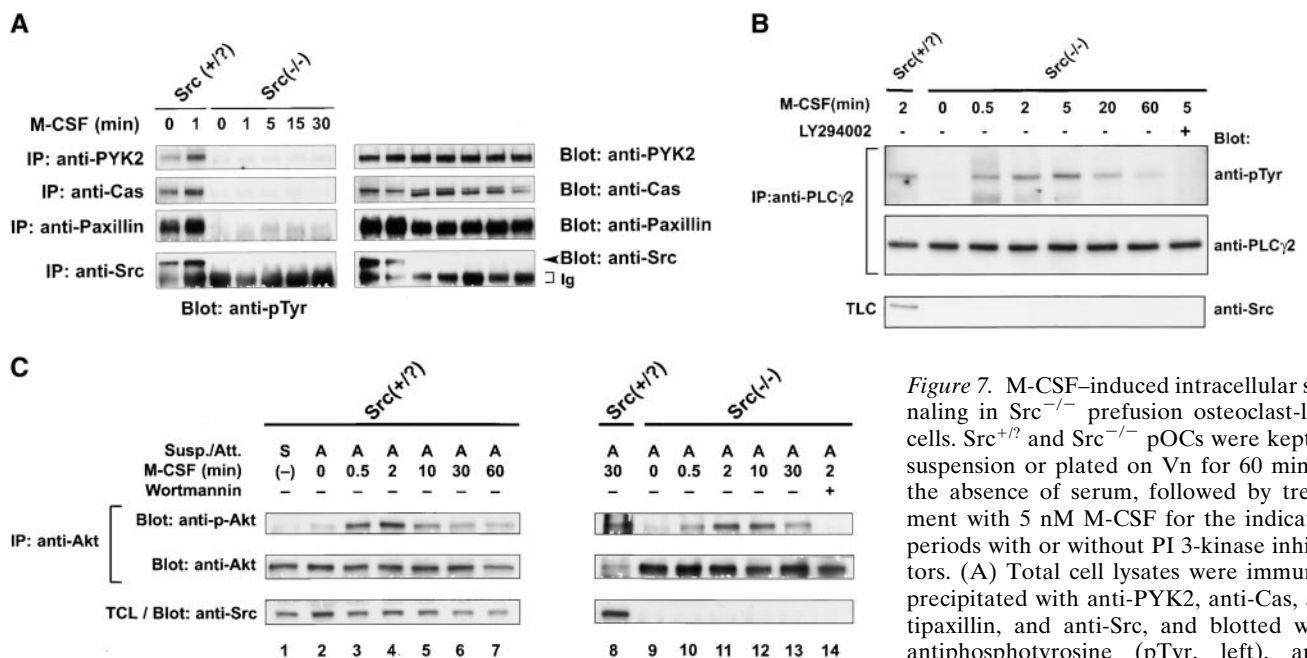

A

Src(+/?) Src(−/−)
M-CSF (min) 0 1   0 1 5 15 30

IP: anti-PYK2 / Blot: anti-PYK2
IP: anti-Cas / Blot: anti-Cas
IP: anti-Paxillin / Blot: anti-Paxillin
IP: anti-Src / ◄Blot: anti-Src] Ig

Blot: anti-pTyr

B

Src(+/?)   Src(−/−)
M-CSF (min) 2   0 0.5 2 5 20 60 5
LY294002 −   − − − − − − +   Blot:

IP:anti-PLCγ2 / anti-pTyr
/ anti-PLCγ2
TLC / anti-Src

C

Src(+/?)   Src(+/?)   Src(−/−)
Susp./Att. S A A A A A A   A   A A A A A A
M-CSF (min) (−) 0 0.5 2 10 30 60   30 0 0.5 2 10 30 2
Wortmannin − − − − − − −   − − − − − − +

IP: anti-Akt { Blot: anti-p-Akt
              Blot: anti-Akt
TCL / Blot: anti-Src

1 2 3 4 5 6 7   8 9 10 11 12 13 14

Figure 7. M-CSF–induced intracellular signaling in Src$^{-/-}$ prefusion osteoclast-like cells. Src$^{+/?}$ and Src$^{-/-}$ pOCs were kept in suspension or plated on Vn for 60 min in the absence of serum, followed by treatment with 5 nM M-CSF for the indicated periods with or without PI 3-kinase inhibitors. (A) Total cell lysates were immunoprecipitated with anti-PYK2, anti-Cas, antipaxillin, and anti-Src, and blotted with antiphosphotyrosine (pTyr, left), anti-PYK2, anti-Cas, antipaxillin, and anti-Src antibodies (right). (B) Lysates were immunoprecipitated with anti–PLC-γ2, blotted with anti-pTyr (top), then with anti–PLC-γ2 antibodies (middle). Part of the total cell lysates were used for immunodetection of c-Src (bottom). (C) Lysates were immunoprecipitated with anti-Akt/PKB, blotted with anti–phospho-Akt/PKB, or anti-Akt/PKB antibodies. S, suspension; A, attached.
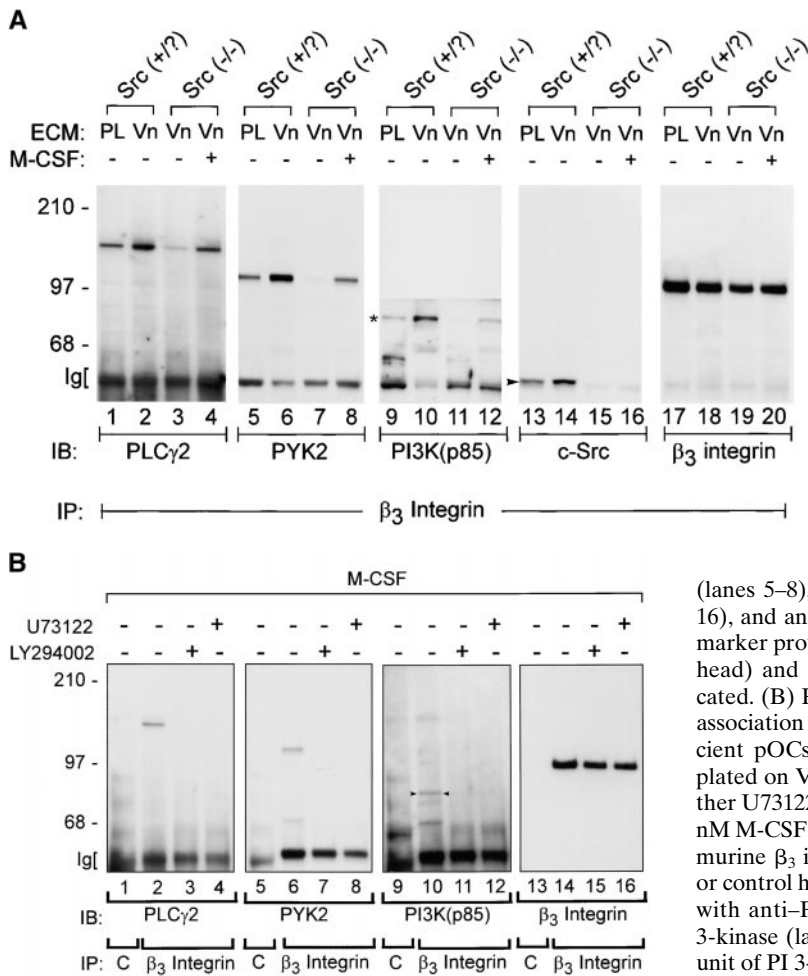
Figure 8. M-CSF–induced association of $\alpha_v\beta_3$ integrin with signaling molecules in Src$^{-/-}$ prefusion osteoclast-like cells. (A) Cell adhesion and M-CSF induce the association of β_3 integrins with signaling molecules in pOCs. Src$^{+/?}$ and Src$^{-/-}$ pOCs (1.5×10^6 cells per condition) were plated on PL- or Vn-coated dishes. After culture for 60 min, Src$^{-/-}$ cells were treated with or without 5 nM M-CSF for 5 min. Total cell lysates were immunoprecipitated (IP) with anti–β_3 integrin antibodies, followed by immunoblotting (IB) with anti–PLC-γ2 (lanes 1–4), anti-PYK2 (lanes 5–8), anti–PI 3-kinase (lanes 9–12), anti–c-Src (lanes 13–16), and anti–β_3 integrin (lanes 17–20). The molecular masses of marker proteins (in kD) are on the left. Positions of c-Src (arrowhead) and of p85 subunit of PI 3-kinase (asterisk) are as indicated. (B) PLC and PI 3-kinase inhibitors block M-CSF–induced association of $\alpha_v\beta_3$ integrin with signaling molecules in Src-deficient pOCs. Src$^{-/-}$ pOCs (1.5×10^6 cells per condition) were plated on Vn as described above, followed by incubation with either U73122 (1 μM) or LY294002 (50 μM) for 40 min, then with 5 nM M-CSF. Lysates were immunoprecipitated with hamster anti–murine β_3 integrin antibodies (lanes 2–4, 6–8, 10–12, and 14–16) or control hamster IgG (lanes 1, 5, 9, and 13), followed by blotting with anti–PLC-γ2 (lanes 1–4), anti-PYK2 (lanes 5–8), anti–PI 3-kinase (lanes 9–12), or anti–β_3 integrin (lanes 13–16). p85 subunit of PI 3-kinase (small arrowheads). C, control hamster IgGs.

skeletal reorganization. We previously reported on the role of PYK2 and p130^{Cas} in the $\alpha_v\beta_3$ integrin–mediated signaling pathways (Duong et al., 1998; Lakkakorpi et al., 1999) and on the involvement of PI 3-kinase in osteoclast adhesion and spreading (Lakkakorpi et al., 1997). Since U73122 blocked the adhesion- and M-CSF–dependent cell spreading of pOCs, we examined the tyrosine phosphorylation levels of PLC-γ1 and 2 in pOCs upon adhesion to Vn. Src$^{+/?}$ or Src$^{-/-}$ pOCs were either left in suspension or plated on Vn-coated dishes. Cell lysates were analyzed by Western blotting with antiphosphotyrosine antibodies after immunoprecipitation. In wild-type cells, PYK2, p130^{Cas}, paxillin, and both PLC-γ1 and 2 became tyrosine phosphorylated paralleling the time course of cell spreading, i.e., peaking at 30 min after plating (Fig. 6 A). These data indicate that PLC-γ1 and 2 are downstream effectors of the integrin-mediated signaling pathway. Furthermore, tyrosine phosphorylation of these molecules after cell adhesion was absent in Src$^{-/-}$ pOCs (Fig. 6 B), supporting the morphological observations shown in Fig. 1. The data thus implicated Src tyrosine kinase as playing an essential role in osteoclast function by mediating integrin-dependent signaling triggered by ligand engagement.

We previously observed tyrosine phosphorylation of paxillin in attached and spread Src$^{-/-}$ OCLs under steady state conditions (Duong et al., 1998). However, in this study, we found that in Src$^{-/-}$ pOCs, paxillin was not tyrosine phosphorylated immediately following adhesion (Fig. 6 B). In order to reconcile these observations, we re-

cultured wild-type or Src$^{-/-}$ pOCs with osteoblastic/stromal MB1.8 cells for 12 h to form OCLs, and compared them with the same number of pOCs both plated on Vn-coated dishes for 60 min. Levels of tyrosine phosphorylated paxillin were analyzed in OCLs, after removal of MB1.8 cells, and pOCs. As shown in Fig. 6 C, paxillin was indeed tyrosine-phosphorylated in Src-deficient OCLs (lane 4) as compared to Src$^{-/-}$ pOCs (lane 3). It should be noted that we consistently observed low yields of Src-deficient OCLs after enzymatic treatment, which is probably due to reduced spreading, as previously reported (Duong et al., 1998). Nevertheless, our observations suggest that in spread Src-deficient OCLs at steady state paxillin is phosphorylated, probably as a consequence of their interaction with the osteoblasts/stromal cells.

M-CSF Induces Tyrosine Phosphorylation of PLC-γ and Activation of PI 3-Kinase in Src-deficient Osteoclasts

As shown above, M-CSF induces spreading in Src$^{-/-}$ prefusion osteoclasts in an $\alpha_v\beta_3$-dependent manner. Therefore, we examined the downstream effectors involved in M-CSF–dependent signaling in the absence of c-Src. Wildtype and Src-deficient pOCs were plated on Vn-coated dishes for 1 h to achieve maximal activation of the adhesion-induced signals, and were then treated with M-CSF at the indicated time (Fig. 7). Although M-CSF appeared to further induce adhesion-dependent tyrosine phosphoryla-
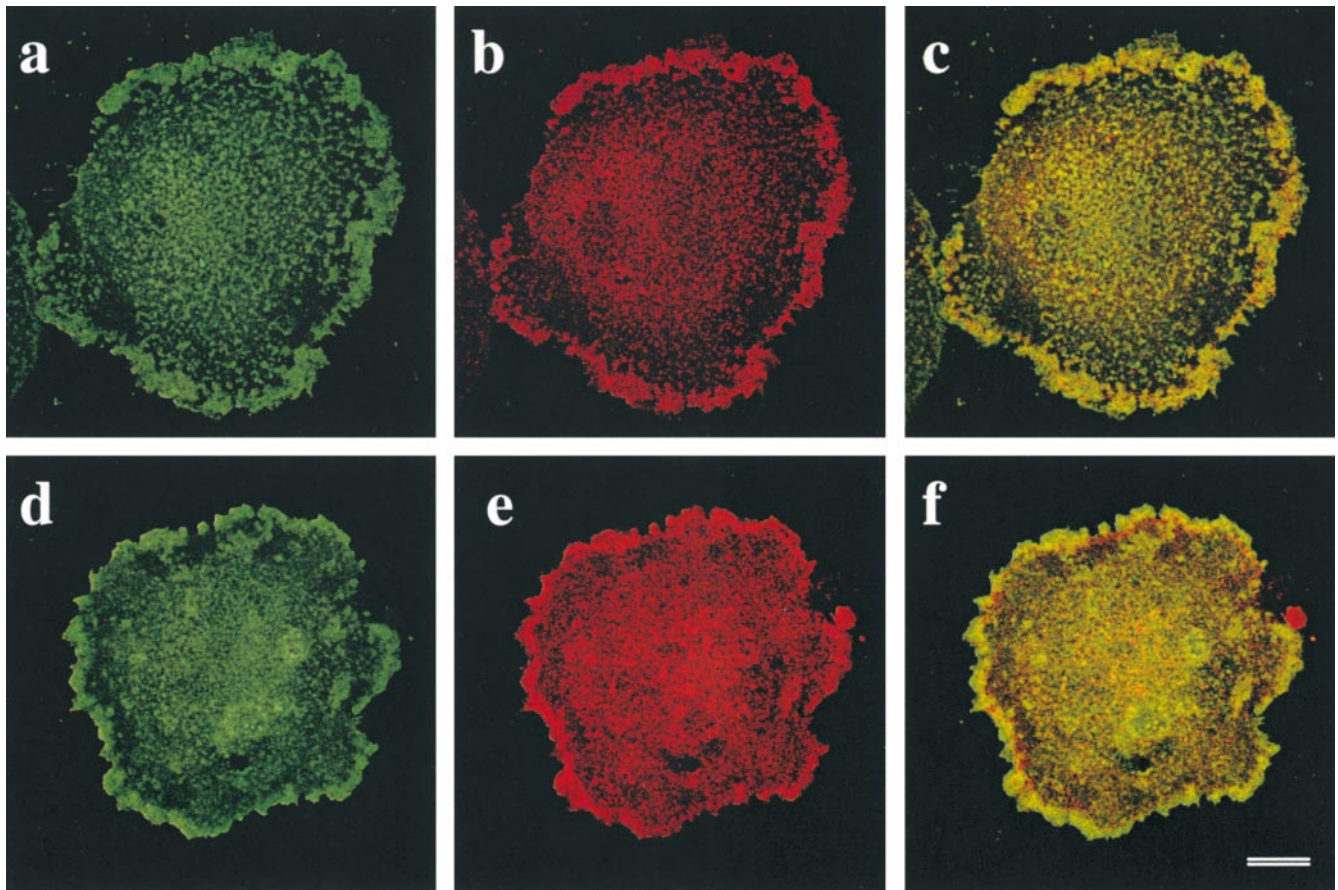
Figure 9. Colocalization of $\alpha_v\beta_3$ integrin and PLC-γ in M-CSF treated Src$^{-/-}$ prefusion osteoclasts. Src$^{-/-}$ pOCs were plated on Vn-coated glass coverslips for 1 hr, then treated with 5 nM M-CSF for 30 min. Cells were fixed double stained with polyclonal anti-β3 integrin and monoclonal anti-PLC-γ1 or PLC-γ2 antibodies. Pseudocolored confocal microscopic images of β3 integrins (a, d, in green) and double staining of PLC-γ1 (b, in red) or PLC-γ2 (e, in red). Colocalization (c, f, in yellow) appears to be more prominent in the adhesion contacts organized at the spreading edge of the cells. Images merged from optical sections of 4.7 μm thickness at the adhesion surface of the cells. Bar, 10 μm.

tion of PYK2, p130^{Cas}, and paxillin in wild-type cells, tyrosine phosphorylation of these molecules was not detected in M-CSF–treated Src-deficient pOCs (Fig. 7 A). In contrast, M-CSF rapidly induced tyrosine phosphorylation of PLC-γ2 (Fig. 7 A) and PLC-γ1 (data not shown) within 0.5 min in these cells, which gradually returned to basal levels after 60 min, suggesting that tyrosine phosphorylation of both PLC-γ isoforms by M-CSF is Src independent. The M-CSF-induced PLC phosphorylation was found to be transient, as compared to that of $\alpha_v\beta_3$-dependent PLC phosphorylation in Src$^{+/?}$ pOCs (Fig. 6 A).

PI 3-kinase activity was previously reported to be required for PLC-γ activation (Falasca et al., 1998; Gratacap et al., 1998). In this study, LY294002, a selective PI 3-kinase inhibitor, inhibited M-CSF–mediated tyrosine phosphorylation of PLC-γ2 (Fig. 7 B), indicating that PI 3-kinase is an upstream mediator of PLC-γ activation in osteoclasts. Given the limitations of this cell system, including relatively small cell numbers and short survival of purified osteoclast-like cells in culture, which precluded direct determination of PI 3-kinase activity, we examined the activation of Akt/PKB as a downstream target of PI 3-kinase in these cells (Downward, 1998). Indeed, in Src$^{-/-}$ pOCs (Fig. 7 C, right) as well as in wild-type cells (Fig. 7 C, left), M-CSF–induced phosphorylation of Akt/PKB,

which was blocked by the PI 3-kinase inhibitors, wortmannin (Fig. 7 C, left), and LY294002 (data not shown). We also noted an increased level of Akt proteins in Src-deficient pOCs (Fig. 7C). Using imaging densitometry, the ratio of Akt protein levels in Src$^{-/-}$ to wild-type pOCs was estimated to be 1.7-fold. Furthermore, the specific activity of tyrosine phosphorylated Akt in wild-type and Src$^{-/-}$ pOCs upon treatment with M-CSF, at peak levels (2 min) relative to basal levels (0 min), were estimated to be 4.4 and 3.9, respectively. This indicated that although Akt proteins appeared to be induced in the absence of c-Src in pOCs, the extent and time course of M-CSF-induced phosphorylation of Akt were similar in wild-type and Src-deficient prefusion osteoclasts (Fig. 7C). Interestingly, the time course of PLC phosphorylation (Fig. 7B) is coincident with M-CSF-induced activation of PI 3-kinase (Fig. 7C). Since LY294002 blocked PLC-γ phosphorylation (Fig. 7B), these data indicated that in Src-deficient pOCs, M-CSF activates PI 3-kinase, which subsequently leads to PLC-γ activation. Taken together, these findings implicate PI 3-kinase and PLC-γ in the M-CSF-dependent cytoskeletal organization in Src-deficient osteoclasts, which further induces ligand engagement of $\alpha_v\beta_3$ integrins, formation of adhesion contacts and cell spreading as shown in Fig. 2 A.
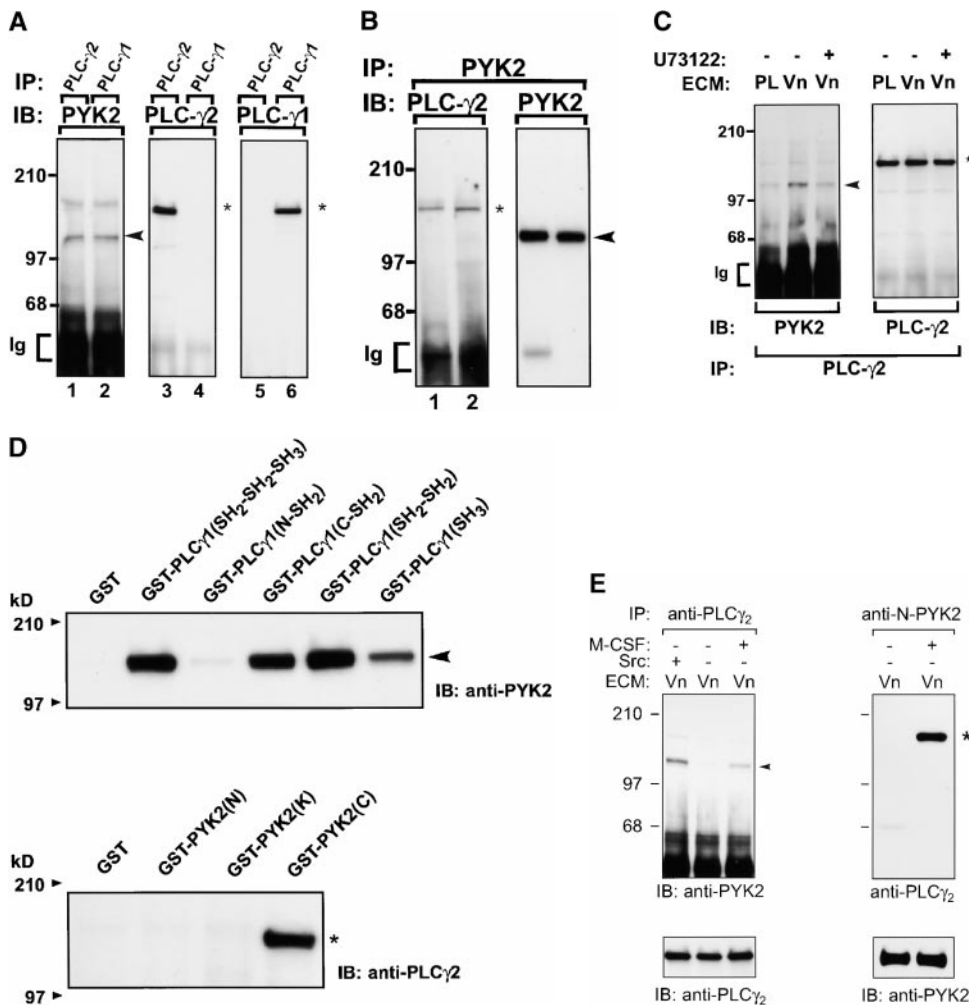
Figure 10. M-CSF–dependent association of PLC-γ2 and PYK2 in osteoclasts. Src[+/?] pOCs were cultured on Vn-coated dishes for 60 min in the absence of serum. (A) Total cell lysates were immunoprecipitated (IP) with anti–PLC-γ2 (lane 1) and anti–PLC-γ1 (lane 2), followed by blotting with anti-PYK2 (left), anti–PLC-γ2 (middle), or anti–PLC-γ1 (right) antibodies. (B) Lysates were immunoprecipitated with anti-PYK2 mAb 11 (lane 1) and anti-PYK2 N-19 antibodies (lane 2), followed by blotting with anti–PLC-γ2 (left) or anti-PYK2 (right). (C) Src[+/?] pOCs were cultured on PL or Vn for 60 min with or without 1 μM U73122. Lysates were immunoprecipitated with anti–PLC-γ2 and blotted with anti-PYK2 (left), and anti–PLC-γ2 (right) antibodies. (D) Lysates of Src[+/?] OCLs were incubated with GST fusion proteins containing NH₂- and COOH-terminal SH2 domains or SH3 domains of PLC-γ1 and blotted with anti-PYK2 antibodies (top) or incubated with GST fusion proteins of NH₂- or COOH-terminal domains or kinase (K) domain of PYK2 and blotted with anti–PLC-γ2 antibodies (bottom). (E) Src^{−/−} pOCs (1.5 × 10⁶ cells per condition) were plated on Vn and treated without and with M-CSF. Adhesion of Src[+/?] pOCs on Vn was used as control. Lysates were immunoprecipitated with anti–PLC-γ2 and blotted with anti-PYK2, followed by anti–PLC-γ2 antibodies (left) or immunoprecipitated with anti-N terminal PYK2 and blotted with anti–PLC-γ2, followed by anti-PYK2 antibodies (right). Molecular weight markers (in kD) are as indicated on the left. Positions of PYK2 (arrowhead) and of PLC-γ (asterisk) are as indicated.

M-CSF–induced Recruitment of Downstream Mediators to β₃ Integrins in Src-deficient Osteoclasts, Is Similar to Adhesion-dependent Recruitment in Wild-Type Cells

Since previous reports demonstrated the association of $\alpha_v\beta_3$ integrins with c-Src and PI 3-kinase in osteoclasts (Hruska et al., 1995; Lakkakorpi et al., 1997), we examined by coimmunoprecipitation with anti–β₃ integrin antibodies the adhesion-dependent association of $\alpha_v\beta_3$ with PLC-γ, PI 3-kinase, c-Src, and PYK2. In pOCs, cell adhesion to Vn increased the association of β₃ integrins with PLC-γ2, PI 3-kinase, PYK2, and c-Src (Fig. 8 A, lanes 1 and 2, 5 and 6, 9 and 10, and 13 and 14, respectively). These data suggest that integrin–ligand engagement induces not only tyrosine phosphorylation of these signaling molecules but also their association with the integrin receptor.

On the other hand, in Src-deficient pOCs plated on Vn, PLC-γ2, PI 3-kinase, and PYK2 were only weakly coimmunoprecipitated with the β₃ integrins (Fig. 8 A, lanes 3, 7, and 11), indicating that Src kinase is important for the adhesion-dependent recruitment of various downstream mediators to the integrin receptor. However, association of $\alpha_v\beta_3$ integrins with PLC-γ2, PI 3-kinase, and PYK2 was promoted in Src^{−/−} pOCs by treatment with M-CSF (Fig. 8 A, lanes 3 and 4, 7 and 8, and 11 and 12). These data suggest that in the absence of c-Src, M-CSF–induced activation of PLC-γ2 and PI 3-kinase was sufficient to further the recruitment of PYK2 to $\alpha_v\beta_3$ receptors independent of tyrosine phosphorylation.

Additional evidence for the role of PLC and PI 3-kinase in the M-CSF–dependent association of $\alpha_v\beta_3$ integrins with their downstream effectors was provided by the fact that either U73122 or LY294002 disrupted the recruitment of PLC-γ2, PYK2, and PI 3-kinase to β₃ integrins in Src-deficient pOCs (Fig. 8 B). These data supported the pharmacological findings suggesting that PI 3-kinase and PLC take part in both, adhesion- and M-CSF-dependent signaling. The findings also suggest that M-CSF modulates $\alpha_v\beta_3$ integrin-dependent signaling via activation of PI 3-kinase and PLC-γ, leading to cytoskeletal reorganization and formation of integrin-associated adhesion contacts in osteoclasts.

To further test the involvement of $\alpha_v\beta_3$ integrins in M-CSF–dependent spreading of Src^{−/−} pOCs, we examined the localization of $\alpha_v\beta_3$ in M-CSF–treated cells. As shown in Fig. 9, β₃ integrins (a and d, in green) colocalized

with F-actin (Fig. 9 b, red) as well as PLC-γ2 (Fig. 9 e, red). Colocalization of β3 integrins and PLC-γ were found in adhesion contacts of the M-CSF-treated Src-deficient pOCs plated on Vn (Fig. 9 c and f, in yellow).

Adhesion- and M-CSF–dependent Association of PYK2 and PLC-γ in Osteoclasts

We next examined which molecular interactions are important for the convergence of the integrin- and M-CSF–dependent signals in prefusion osteoclasts in the absence of c-Src. Since we were previously unable to demonstrate stable interactions of PYK2 and PI 3-kinase in OCLs (Duong et al., 1998), we examined the association of PYK2 with PLC-γ in these cells. Both anti–PLC-γ1 and 2 antibodies coprecipitated PYK2 (Fig. 10 A) and anti-PYK2 antibodies pulled down PLC-γ2 (Fig. 10 B), supporting the in situ association of the two proteins in OCLs. Furthermore, upon adhesion to Vn a stronger association of PYK2 and PLC-γ was observed than in cells plated on PL (Fig. 10 C). Moreover, in the presence of U73122 the association of PYK2 and PLC-γ2 was reduced to the level observed in cells on PL (Fig. 10 C). These findings suggest that in osteoclasts, both integrin-dependent activation of PYK2 and PLC-γ2 and the phospholipase activity itself might be important for the stable interaction between these molecules.

To partially characterize the domains of PLC-γ which mediate binding to PYK2, GST fusion proteins encoding the NH_2- and COOH-terminal SH2 domains or the SH3 domain of PLC-γ1 were incubated with lysates prepared from OCLs. GST fusion protein containing the COOH-terminal SH2 domain and the SH3 domain of PLC-γ bound to PYK2 from OCL lysates (Fig. 10 D, top), suggesting that PLC-γ could bind to either a tyrosine phosphorylated moiety or to a proline-rich region of PYK2. Conversely, the COOH-terminal domain containing proline-rich regions of PYK2 was found to bind to PLC-γ2 (Fig. 10 D, bottom), supporting the above observations on both the adhesion (phosphorylation)-dependent association of PYK2 and PLC-γ, as well as their constitutive interaction. Additional studies will be conducted to further analyze the structural features that are important for the interaction of these two molecules.

Since we found that both integrin-dependent activation of PYK2 and PLC-γ2 and its phospholipase activity were important for their interaction in wild-type pOCs, we thus proceeded to examine the direct interaction of PYK2 and PLC-γ2 in M-CSF–treated Src-deficient pOCs. Although PYK2 is not tyrosine phosphorylated in $Src^{-/-}$ pOCs (Fig. 7 A), direct interaction of PYK2 and PLC-γ2 was induced in response to M-CSF (Fig. 10 E). This observation was reproduced in three separate experiments and supports the role of PLC activity in the integrin- and M-CSF–mediated association with their downstream mediators (Fig. 8, A and B). Furthermore, these data suggest that the direct association of PYK2 and PLC-γ might play an important role in both M-CSF– and adhesion-dependent signaling pathways in prefusion osteoclasts.

Discussion

Src kinases play an important role in cell adhesion and migration, in cell cycle control, and in cell proliferation and differentiation (Thomas and Brugge, 1997). Moreover, novel roles for Src kinases in the control of cell survival and angiogenesis have recently emerged (Schlessinger, 2000). In this study, we examined integrin- and M-CSF–mediated signaling pathways involved in the adhesion and migration of osteoclast precursors, using $Src^{+/?}$ and $Src^{-/-}$ pOCs formed in vitro. The findings indicate that c-Src is essential for integrin-initiated signaling in these cells upon ligand engagement, since the absence of c-Src causes impairment in cell spreading associated with significant reduction in tyrosine phosphorylation of several adhesion/signaling molecules including PYK2, p130^{Cas}, paxillin, and PLC-γ. The involvement of Src family kinases in integrin-mediated signaling pathway has been reported in $Src^{-/-}$ $Yes^{-/-}Fyn^{-/-}$ triple mutant cells (Klinghoffer et al., 1999) and macrophages derived from $Hck^{-/-}$ $Fgr^{-/-}Lyn^{-/-}$ triple mutant mice (Meng and Lowell, 1998). Triple deletions of Src family kinases are required to block the integrin-dependent signals in these cells, probably due to functional overlap. On the other hand, in Src-deficient fibroblasts, the vitronectin receptor-mediated traction forces during cell migration were recently demonstrated to be selectively modulated by c-Src (Felsenfeld et al., 1999).

Osteoclasts abundantly express c-Src, as well as very low levels of c-fyn, c-yes, and c-lyn (Horne et al., 1992). However, the absence of c-Src is sufficient to abolish bone resorption in vivo, without reducing osteoclast number (Soriano et al., 1991), suggesting that these members of the Src kinase family do not compensate for the absence of c-Src in osteoclast function, both in vivo and in vitro (Horne et al., 1992). Indeed in $Src^{-/-}$ pOCs, we found no change in protein levels of c-yes and c-lyn, and a very small increase (<2-fold) in c-fyn expression, and could not detect these members of c-Src family kinases in immunoprecipitates of $α_vβ_3$ integrins (data not shown). Nevertheless, we show in this study that c-Src is not required for M-CSF–induced cytoskeletal reorganization in prefusion osteoclast-like cells. M-CSF induces cell spreading and migration, along with tyrosine phosphorylation of PLC-γ2 in $Src^{-/-}$ pOCs, although it did not induce tyrosine phosphorylation of PYK2 and p130^{Cas} under the same conditions. We previously observed tyrosine phosphorylation of paxillin in attached and spread $Src^{-/-}$ OCLs (multinucleated osteoclast like cells) under steady state conditions (Duong et al., 1998). In this study, we examined adhesion-mediated signaling immediately following the attachment of $Src^{-/-}$ pOCs and found that paxillin is not tyrosine phosphorylated during initial adhesion process. To resolve this apparent inconsistency, we re-cultured $Src^{-/-}$ pOCs with osteoblastic/stromal cells to form OCLs, and found that paxillin was indeed tyrosine phosphorylated in these attached cells under steady state conditions. This observation suggests that in OCLs paxillin is phosphorylated by an alternative kinase probably in response to stimuli received from osteoblasts/stromal cells.

Our observations are consistent with a recent report showing that PDGF-mediated signaling is similar in $Src^{-/-}$ $Yes^{-/-}Fyn^{-/-}$ triple mutant fibroblasts and the wild-type controls (Klinghoffer et al., 1999). In addition, our in vitro findings of M-CSF–induced cell spreading and migration of $Src^{-/-}$ prefusion osteoclasts could be relevant to in vivo observations on Src-deficient mice, where osteoclasts are multinucleated and adhere to the bone surface. The ability

of Src-deficient osteoclasts to spread and migrate in vivo (Boyce et al., 1992) could reflect the influence of M-CSF or other growth factors. Moreover, transgenic expression of kinase-deficient Src in Src$^{-/-}$ mice rescued osteoclast function, indicating that Src may function in part as an adaptor to recruit downstream signaling molecules (Schwartzberg et al., 1997). Our findings apparently differ from a previous report showing that M-CSF did not induce cell spreading in Src-deficient osteoclasts derived from Src knockout mice (Insogna et al., 1997). The difference could be due to use in that study of adherent multinucleated primary osteoclasts in the presence of serum and bone marrow stromal cells. The present study used purified prefusion osteoclast-like cells under serum-free condition, in which M-CSF–mediated signaling could be enhanced.

The data presented here support the role of PLC-γ in integrin-dependent regulation of cytoskeletal organization. This is supported by induction of PLC-γ tyrosine phosphorylation upon cell adhesion and inhibition of cell spreading in wild-type osteoclasts by a PLC inhibitor. These observations are consistent with previous studies showing that integrin–ECM interactions induce tyrosine phosphorylation of PLC-γ1 (Langholz et al., 1997) and PLC-γ2 (Asselin et al., 1997). It was also recently reported that phosphorylation of PLC-γ1 at the tyrosine residue 783 is important for regulation of cytoskeletal organization in fibroblasts (Yu et al., 1998; Pei and Williamson, 1998), while PLC-γ1 can serve as a substrate of c-Src in in vitro kinase assays (Liao et al., 1993; Nakanishi et al., 1993). Furthermore, PLC-γ1–null fibroblasts exhibit a more round-up morphology than their normal counterparts (Ji et al., 1997). Taking advantage of the crucial role of c-Src in osteoclasts, we demonstrated that in these cells PLC-γ is downstream of c-Src, since adhesion does not induce tyrosine phosphorylation of PLC-γ1 and 2 in Src-deficient pOCs.

This study points to interactions between adhesion- and growth factor–initiated signal transduction, which seem to play a role in cell spreading and migration. There are several possible mechanisms for synergy between adhesion and growth factor signaling pathways (Schwartz and Ingber, 1994; Yamada and Miyamoto, 1995), for example activation of common downstream effectors. We suggest that in prefusion osteoclasts PLC-γ is one of the downstream molecules, activated by adhesion- and M-CSF–dependent signals, that lead to cytoskeletal reorganization. PLC-γ is activated either by cell attachment in a Src dependent manner or by M-CSF-treatment which is not Src dependent. The role of PLC is supported by pharmacological evidence showing that PLC inhibitors block both adhesion- and M-CSF–induced cell spreading. Previous studies have implicated MAP kinases as candidates for this cross-signaling (Chen et al., 1994; Zhu and Assoian, 1995); however, in osteoclasts, M-CSF did not activate MAP kinases ERK1 and 2 in Src$^{-/-}$ pOCs, and the MAP kinase kinase inhibitor, PD98059, had little effect on M-CSF–induced cell spreading of Src-deficient prefusion osteoclasts.

Another likely mechanism for the synergy between adhesion- and growth factor–mediated signaling pathways is the physical interaction (clustering) of key components of both pathways, allowing the convergence of the two (Thomas and Brugge, 1997; Giancotti and Ruoslahti, 1999). Coclustering of integrins and growth factor receptors appears to require association with the cytoskeleton

and recruitment of downstream signaling molecules. Aggregation of these molecules has been thought to bring both adhesion- and growth factor-mediated signaling closer to a threshold of manifest activity (Giancotti and Ruoslahti, 1999). Recent reports have documented the physical interaction of α$_v$β$_3$ with the insulin, PDGF or VEGF receptors in fibroblasts (Woodard et al., 1998; Soldi et al., 1999). More recently, FAK was demonstrated to be an important proximal link between PDGF and EGF receptors and β1 integrins during fibroblast chemotactic migration (Sieg, et al., 2000). Interestingly, for chemotactic cell motility FAK kinase activity is dispensable, while phosphorylation at FAK Y397, the Src-kinase binding site, and the integrity of the actin cytoskeleton are required for PDGF/EGF- and integrin-mediated cell migration (Sieg, et al., 2000).

In the case of prefusion osteoclasts, our data suggest that M-CSF can modulate the localization of α$_v$β$_3$ and its interaction with downstream effectors in a c-Src–independent manner. This is supported by the following findings: first, M-CSF–induced cell spreading of Src$^{-/-}$ pOCs depends on attachment to Vn; second, echistatin, an α$_v$β$_3$ integrin antagonist, blocks M-CSF–induced cell spreading; third, in M-CSF–treated Src$^{-/-}$ pOCs, β$_3$ integrin localizes to adhesion contacts along with PLC; and fourth, association of α$_v$β$_3$ with PYK2, PI 3-kinase, and PLC-γ in Src$^{-/-}$ prefusion osteoclasts is M-CSF dependent and PYK2 binds directly to PLC-γ. These findings suggest that activation of M-CSF receptors result in the recruitment of intracellular signaling molecules to α$_v$β$_3$ integrins at adhesion contacts. Furthermore, in Src-deficient cells, M-CSF induces the association of β$_3$ integrin engaged by its extracellular ligand with signaling molecules including PI 3-kinase, PLC-γ, and PYK2, independent of PYK2 tyrosine phosphorylation. These interactions are blocked by PLC or PI 3-kinase inhibitors. Therefore, our data suggest that activation by either integrin ligands or growth factors results in the physical recruitment of key components of these pathways to adhesion contacts. On the other hand, we could not convincingly demonstrate the presence of M-CSF receptors in the α$_v$β$_3$-associated immunocomplexes (data not shown). We are presently investigating further the possible physical association of M-CSF receptor with α$_v$β$_3$ integrin in osteoclasts during chemotactic migration.

The observations on PI 3-kinase are consistent with previous reports showing that growth factor receptors, e.g., PDGF (Kinashi et al., 1995), thrombopoietin (Zauli et al., 1997), insulin (Guilherme et al., 1998), EGF (Adelsman et al., 1999), and VEGF (Soldi et al., 1999) stimulate integrin-mediated cell adhesion through a PI 3-kinase–dependent pathway. In the case of osteoclasts, the association of α$_v$β$_3$ integrins with PI 3-kinase has been reported (Hruska et al., 1995; Lakkakorpi et al., 1997). Present findings suggest that PLC-γ is a downstream effector of PI 3-kinase, involved in the regulation of integrin-dependent signaling by growth factors. Consistent with these observations, Shibayama et al. (1999) reported recently that U73122 blocks IL-3–induced α$_4$β$_1$ and α$_5$β$_1$ integrin activation in Baf3 cells.

An obvious question is how M-CSF–dependent activation of PI 3-kinase and PLC-γ modulate integrin function. FAK was demonstrated to bind to peptides that mimic the β$_1$ integrin cytoplasmic domains (Shaller et al., 1995). In addition, Plopper et al. (1995) reported that RGD-coated

beads pulled down the molecular complex that contains FAK, c-Src, and PLC-γ in capillary endothelial cells. Recently, Zhang et al. (1999) reported that PLC-γ1 can associate with FAK. This association is mediated by tyrosine-397 in FAK and the COOH-terminal SH2 domain of PLC-γ1 and is dependent on cell adhesion. We found that PYK2, a member of the FAK family kinases, is highly expressed in osteoclasts and is tyrosine phosphorylated in a c-Src–dependent manner upon $\alpha_v\beta_3$-mediated adhesion (Duong et al., 1998). In addition, PYK2 localizes to podosomes, the primary adhesion structures in osteoclasts (Duong et al., 1998). In this study, PLC-γ was found to associate with PYK2 independent of PYK2 phosphorylation, probably via the SH3 domain of PLC-γ and the proline-rich domains toward the COOH-terminal region of PYK2. Importantly, this interaction was further enhanced upon osteoclast adhesion to Vn, possibly via interaction of the COOH-terminal SH2 domain of PLC-γ with tyrosine-402 in PYK2 (Schlaepfer et al., 1999). This interaction is sensitive to the PLC-γ inhibitor. Taken together, these data suggest that in osteoclasts either integrin- or M-CSF–mediated signals result in recruitment of PYK2 and PLC-γ to the integrin-associated complex at adhesion sites. Furthermore, our data also suggest that PYK2 may function as an adaptor recruiting other integrin-associated molecules, including p130^{Cas} and PLC-γ, during M-CSF-induced Src$^{-/-}$ osteoclast spreading and migration. In part, this observation is supported by a previous study in which kinase-deficient c-Src was implicated to function as an adaptor, when its transgenic expression rescued osteoclast function in Src$^{-/-}$ mice (Schwartzberg et al., 1997).

Questions that remain to be answered relate to how PI 3-kinase and PLC-γ can mediate cell spreading and migration in response to growth factors and cytokines. PI 3-kinase-mediated activation of PLC- was suggested to be important for PLC membrane targeting (Falasca et al., 1998). One candidate molecule might be PKC, which is activated by DAG, a product of PLC-γ (Kolanus and Seed, 1997). In FG human carcinoma cells, Klemke et al. (1994) demonstrated a link between activation of EGF receptor tyrosine kinase, PKC, and integrin-dependent cell spreading. The pleckstrin homology domain of PLC-γ was shown to preferentially recognize 3-phosphorylated phosphoinositides, including PtdIns(3)P, PtdIns(3,4)P$_2$, and PtdIns(3,4,5)P$_3$ (PIP3) and to lesser extent PtdIns(4,5)P$_2$ (PIP2) (Falasca et al., 1998; Kavran et al., 1998). Recent studies revealed the cytoplasmic distribution of PtdIns(3)P to endosomes, and PtdIns(3,4)P$_2$ and PIP3 to plasma membranes, and implicated roles of these PI 3-kinase products in regulation of various processes, including endosome fusion and motility, phagocytosis, pinocytosis, regulated exocytosis, and cytoskeletal organization (Czech, 2000). Recently, the local concentration of PIP2 was suggested to control adhesion strength of the actin-based cytoskeleton to plasma membrane, which also define cell shape and cell movement (Raucher et al., 2000). Interestingly, the localized adhesion energy in NIH3T3 cells was shown to be reduced by either EGF or PDGF, known stimuli that activate PLC-γ, and this reduction could be blocked by U73122 (Raucher et al., 2000). Together, these studies suggest that PLC-γ and PI 3-kinase, which mediate the cytoskeletal structure by changing local concentrations of

PIP2, PIP3, DAG, and calcium, could indirectly modulate integrin function.

In summary, we have demonstrated that in prefusion osteoclasts: (a) c-Src is essential for integrin "outside-in" signaling; (b) c-Src is not necessary for M-CSF–mediated cytoskeletal reorganization; (c) PLC-γ is a common downstream mediator for adhesion and growth factor signals; and (d) M-CSF–initiated signaling modulates the $\alpha_v\beta_3$ integrin–ligand interaction and the recruitment of signaling molecules to adhesion structures, possibly via PLC-γ activation.

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