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Activated c-Fms recruits Vav and Rac during CSF-1-induced cytoskeletal remodeling and spreading in osteoclasts

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Abstract

Colony-stimulating factor-1 (CSF-1) induces osteoclast spreading that requires activation of c-Src and phosphatidyl inositol 3-kinase (PI3-K), both of which are recruited to activated c-Fms, the CSF-1 receptor. The present report provides evidence that the hemopoietic guanine nucleotide exchange factor (GEF), Vav, and its target GTPase, Rac, lie downstream from this initial signaling complex. CSF-1 treatment of osteoclast-like cells induced translocation of Vav to the plasma membrane, an increase in its phosphotyrosine content, and a concomitant decline in the amount of phosphoinositol 4,5-bisphosphate bound to Vav, changes known to induce Vav's GEF activity. CSF-1 induced the association of Vav and Rac and increased Rac's GTPase activity. CSF-1 also induced rapid translocation of Rac to the periphery of spreading neonatal rat osteoclasts where it co-localized primarily with Vav3 and to a lesser extent with Vav1. Wortmannin, an inhibitor of PI3-K, blocked CSF-1-induced Rac translocation and prevented CSF-1-induced spreading and actin reorganization in osteoclasts. CSF-1-induced osteoclast spreading was not significantly reduced in osteoclasts isolated from Vav1 knock-out mice and Vav1 knock-out mice had normal bone density. Microinjection of constitutively active Rac, but not constitutively active Cdc42 or RhoA, induced lamellipodia formation and osteoclast spreading, mimicking the effects of CSF-1. Dominant-negative Rac blocked CSF-1-induced osteoclast spreading, whereas neither dominant-negative Cdc42 nor C3, an inhibitor of RhoA, affected the response to CSF-1. These data demonstrate that Vav and Rac lie downstream from activated PI3-K in CSF-1-treated osteoclasts and that Rac is required for CSF-1-induced cytoskeletal remodeling in these cells.

Keywords: Osteoclast; CSF-1; Rac; Cdc42; Rho; Vav; PI3-kinase

Introduction

Bone remodeling is a complex, highly orchestrated process that is initiated by a cycle of bone resorption, mediated, at the cellular level, by osteoclasts [1]. A distinguishing feature of the resorbing activity of osteoclasts is their ability to move along the bone surface. After excavating a resorbing pit, the sealing zone of an osteoclast is disassembled and the cells move to a new site of resorption. The signals that attract osteoclasts to new sites of resorption are unknown. One possible signal may be colony-stimulating factor-1 (CSF-1).

CSF-1 clearly affects osteoclastogenesis and must therefore have osteoclast progenitors as one of its targets in bone. However, the CSF-1 receptor, c-Fms, is most highly expressed on mature osteoclasts [2]. We and others have reported that CSF-1 induces cell spreading, motility, and actin reorganization in mature osteoclasts [3–5]. The signaling cascade that regulates these cytoskeletal events is not clear. C-src appears to be required for CSF-1's cytoskeletal effects because mature osteoclasts derived from $src^{-/-}$ mice do not spread in response to CSF-1 [6]. However, when plated on vitronectin, $src^{-/-}$ pre-osteoclasts can respond to CSF-1 [7]. Phosphatidylinositol 3-kinase (PI3-K) is also important for this process because inhibition of this enzyme prevents CSF-1-induced osteoclast spreading and motility [5].

Available evidence suggests that the substrate, phosphoinositol 4,5-bisphosphate (PIP_2), and product, phosphoinositol 3,4,5-triphosphate (PIP_3), of PI3-K play important signaling roles. For example, binding of these phospholipids to a

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conserved protein motif called the pleckstrin homology (PH) domain in guanine nucleotide exchange factors (GEFs) markedly influences the activity of these molecules [8]. Thus, the PI3-K substrate, PIP_2, when bound to the PH domain of the GEF, Vav, inhibits its activity, whereas binding of the product, PIP_3, activates Vav. In this way, activation of PI3-K results in activation of Vav in part by facilitating exchange of an inhibitory molecule PIP_2, for a stimulatory molecule PIP_3, on Vav's PH domain.

Vav and other GEFs lie upstream of the Rho GTPase family of proteins Rho, Rac, and Cdc42 [9–13]. The Rho GTPase family of proteins is known to be critically important in regulating the cytoskeleton [13]. In macrophages, activated Rac causes the development of lamellipodia, broad apron-like cytoplasmic extensions, whereas activated Cdc42 stimulates filopodia formation, small finger-like or spike-like cytoplasmic extensions [14]. Activation of Rho causes cytoplasmic retraction [14].

In the current study, we sought to determine whether the Rho GTPases participate in mediating CSF-1's cytoskeletal effects in osteoclasts. We report that the GTPase Rac is required for CSF-1-induced osteoclast spreading and that Vav and Rac appear to lie downstream from PI3-K in a signaling cascade that leads from activated c-Fms to the actin cytoskeleton in these cells.

Methods

Materials

Recombinant human colony-stimulating factor-1 (CSF-1) was a generous gift from Genetics Institute (Cambridge, MA). Alpha-MEM cell culture medium and fetal bovine serum were from Sigma (St. Louis, MO). Antisera to phosphoinositol 4,5-bisphosphate (PIP_2) [15] was kindly provided by Kiyoko Fukami, PhD, Department of Molecular Oncology, University of Tokyo, Tokyo, Japan. Wortmannin and FluorSave were purchased from Calbiochem (La Jolla, CA). Antibodies to the p85 subunit of PI3-K, c-Fms, Rac, Cdc42, phosphotyrosine, and Vav were from Upstate Biotechnology (Lake Placid, NY). Anti-Rho and anti phospho-Vav2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Vav1 and anti-phospho-Vav3 antibodies were from Biosource International (Camarillo, CA). Rhodamine–phalloidin, Alexa Fluor anti-mouse, and anti-rabbit antibodies were from Molecular Probes (Eugene, OR). Etched gridded coverslips were from BELLCO Glass, Inc. (Vineland, NJ).

Osteoclasts and osteoclast-like cells

Mature osteoclasts were isolated from neonatal rat or mouse bones just before use as previously described [6]. Osteoclast-like cells (OCLs) were generated in vitro by co-culturing murine osteoblasts and bone marrow cells on 10-cm tissue culture plates as previously reported [6]. Briefly, primary murine osteoblasts were obtained by serial collagenase/dispase digestion of neonatal CD1 mouse calvariae and plated at an initial density of 2.5×10^4 cells/cm^2 for co-culture. Bone marrow cells were prepared by flushing the marrow from the tibiae and femurs of 7-week old CD1 mice. Nucleated marrow cells were plated at an initial density of 1.5×10^5 cells/cm^2. Co-cultures were grown in α-MEM with 10% FCS, 1% penicillin/streptomycin, 1% L-glutamine, 20 mM HEPES, pH 7.36 containing 10^{-8} M 1,25-dihydroxyvitamin D_3 and 10^{-6} M prostaglandin E_2, with a media change every other day for 6 days. Contaminating mononuclear cells were removed by treating with 5 mM EDTA for 10 min. Approximately 90% of the cellular material derived from these purified cultures is from OCLs. Purified OCLs were directly lysed with TRIzol® (Invitrogen, Carlsbad, CA) for RNA extraction or with lysis buffer for immunoprecipitation and/or western blotting experiments as previously described [5].

Confocal immunocytochemistry

Rat osteoclasts were isolated and allowed to attach to coverslips for 3 h in α-MEM containing 10% FBS. Cells were treated with vehicle or 2.5 nM CSF-1 for the indicated times, fixed in 3.7% formaldehyde for 10 min and washed. For actin labeling, coverslips were incubated in rhodamine–phalloidin for 20 min, washed and mounted in FluorSave. Cells were examined using a Zeiss LSM 510 confocal imaging system. For immunostaining, coverslips were incubated with anti-Rho, Rac, Cdc42, and phospho-Vav antibodies at 4°C overnight, then incubated with Alexa anti-mouse and anti-rabbit antibodies for 1 h, washed and mounted in FluorSave.

Immunoprecipitation and immunoblotting

To detect Vav tyrosine phosphorylation and Vav-associated PIP_2, Vav protein was immunoprecipitated from 500 μg of OCL lysates prepared from cells treated either with vehicle or 2.5 nM CSF-1 for 5 or 10 min. After SDS–PAGE and *trans*-blotting, nitrocellulose membranes were immunoblotted with anti-phosphotyrosine antibody or a monoclonal anti-PIP_2 antibody. In some experiments, OCLs were pretreated with 50 nM wortmannin before exposure to CSF-1. To detect CSF-1-dependent association of Vav and Rac, Rac was immunoprecipitated from lysates prepared from OCLs treated for various times with 2.5 nM CSF-1 and analyzed by western blotting for Vav. The blot was then stripped and reprobed for Rac to ensure that equivalent amounts of Rac were immunoprecipitated in each lane.

Rac and Cdc42 activation assay

Rac and Cdc42 activation were measured using a commercial kit purchased from Upstate Biotechnology Inc. Briefly, purified OCLs were stimulated with vehicle or 2.5 nM CSF-1 for 2 min. Cells were lysed in Mg lysis/wash buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA and 10% glycerol, containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium fluoride, and 1 mM sodium orthovanadate). Cell lysates were clarified by centrifugation at 12,000×*g* for 10 min. Protein concentrations were estimated using Bradford protein assay reagents (Bio-Rad, Hercules, CA). Cell lysates (1 mg/ml) were incubated with 10 μg of p21-activated kinase (PAK-1) PBD agarose and the reaction mixture gently rocked at 4°C for 60 min. The agarose beads were then washed 3 times and resuspended in 2× Laemmli sample buffer. Proteins bound to PAK-1 were separated by SDS–PAGE, transferred to nitrocellulose, and probed with monoclonal anti-Rac or anti-Cdc42 antibodies.

Microinjection

Authentic rat osteoclasts were plated onto gridded coverslips for 3 h in α-MEM containing 10% FBS. The FBS concentration was then reduced to 2% for an additional 2 h before microinjection. Plasmids encoding GST fusion proteins for constitutively active (V14) Rho, constitutively active (L61) Cdc 42, constitutively active (V12) Rac, C3 transferase, and dominant-negative (N17) Cdc42 were kindly provided by Dr. Alan Hall (MRC Laboratory for Molecular Cell Biology and Cell Biology Unit, University College, London). Fusion proteins were prepared using published methods [16]. The microinjections of V12Rac and N17Rac were performed using an Eppendorf microinjector 5242/micromanipulator 5171. The cells were injected with fusion proteins (2 μg/μl) at pressures of P1 > 3000, P2 = 100, and P3 = 50. The microinjections of V14Rho (2 μg/μl), C3 transferase (2 μg/ml), L61Cdc42 (2 μg/μl), and N17Cdc42 (2 μg/μl) were performed using an Eppendorf FemtoJet/Injectman. Injections of these proteins were accomplished using the following injection parameters, Pi = 300, Ti = 0.1, Pc = 100. After microinjection, cells were allowed to recover for 30 min and then treated with either vehicle or 2.5 nM CSF-1 for 20 min. Images of microinjected cells were recorded before injection (−30′), 30 min after injection (0′), and 10 min (10′) and 30 min (30′) after treatment with either vehicle or 2.5 nM CSF-1 using a CCD camera. Changes in cell area were quantitated using NIH Image software version 1.62f and expressed as percent change from basal. *P* values are referent to changes in cells injected with GST alone.