M-CSF Regulates the Cytoskeleton via Recruitment of a Multimeric Signaling Complex to c-Fms Tyr-559/697/721*

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M-CSF is known to induce cytoskeletal reorganization in macrophages and osteoclasts by activation of phosphatidylinositol 3-kinase (PI3K) and c-Src, but the detailed mechanisms remain unclear. We find, unexpectedly, that tyrosine (Tyr) to phenylalanine (Phe) mutation of Tyr-721, the PI3K binding site in c-Fms, fails to suppress cytoskeletal remodeling or actin ring formation. In contrast, mutation of c-Fms Tyr-559 to Phe blocks M-CSF-induced cytoskeletal reorganization by inhibiting formation of a Src Family Kinase SFK-c-Cbl-PI3K complex and the downstream activation of Vav3 and Rac, two key mediators of actin remodeling. Using an add-back approach in which specific Tyr residues are reinserted into c-Fms inactivated by the absence of all seven functionally important Tyr residues, we find that Tyr-559 is necessary but not sufficient to transduce M-CSF-dependent cytoskeletal reorganization. Furthermore, this same add-back approach identifies important roles for Tyr-697 and Tyr-721 in collaborating with Tyr-559 to recruit a multimeric signaling complex that can transduce signals from c-Fms to the actin cytoskeleton.

Cell motility is a dynamic process consisting of repeated cycles of protrusion of filopodia and lamellipodia at the leading front, adhesion of the protruding edge to the substratum via focal complexes, retraction of cytoplasmic actomyosin, and finally release from contact sites at the tail of the cell (1–3). The locomotory apparatus of most cells works against cell-to-substratum adhesions. In macrophages and osteoclasts (OCs), their differentiated progeny, integrin-mediated contacts to the substratum, are called podosomes (4, 5). In contrast to the more stable focal adhesions, podosomes are very dynamic structures that assemble and disassemble rapidly (6, 7), reflecting the motile nature of OCs and their myeloid precursors.

In migrating macrophages and OCs, podosomes are confined to the periphery of the cell, where their reorganization results in lamellipodia formation, a process dependent on de novo actin assembly by the Arp2/3-Ena-Wiskott-Aldrich syndrome protein complex (8). Activation of actin polymerization is regulated by Rac GTPase acting through one or more members of the Wiskott-Aldrich syndrome protein family (9, 10). The fact that podosome formation, macrophage chemotaxis, and osteoclast movement on bone are impaired by mutation of the Wiskott-Aldrich syndrome protein (11), inhibition of Rac activity (12), or small interfering RNA suppression of the actin-related protein 2/3 complex (13, 14) attests to the importance of actin assembly in macrophages and OC function.

Macrophages and OCs both respond to chemotactic stimuli initiated by M-CSF. Re-addition of M-CSF to quiescent BAC1.2F5 macrophages stimulates rapid cytoskeletal reorganization and cell motility within a few minutes, followed by chemotactic migration up a gradient of diffusing cytokine (15). Exposure to the cytokine also increases cell spreading, membrane ruffling, and motility of osteoclasts (12, 16) and macrophages (16).

M-CSF binds to its transmembrane receptor tyrosine kinase (RTK) c-Fms and induces receptor autophosphorylation at seven tyrosine residues within the cytoplasmic domain (17). Several Src homology 2 domain-containing molecules are recruited to the phospho-Tyr residues upon M-CSF binding and initiate different signaling cascades that lead to cell proliferation, differentiation, or cytoskeletal reorganization (17). The identity of the molecules binding the cytoplasmic domain of c-Fms has been determined by expressing wild-type (WT) and tyrosine (Tyr) to phenylalanine (Phe) mutated forms of the RTK in various cell lines. The resulting model suggests that c-Fms Tyr-559 and Tyr-721 bind Src Family Kinases (SFKs) and phosphatidylinositol 3-kinase (PI3K), respectively, whereas Tyr-697 recognizes Grb2 and Tyr-974 is a binding site for c-Cbl (18–20).

Although the detailed signaling cascade activated by M-CSF is not yet fully understood, PI3K and SFKs are recruited to c-Fms and regulate migration of macrophages and OCs. Migration of PI3Kγ- or PI3Kα-null macrophages is impaired in response to the cytokine (21, 22). Similarly, wortmannin, a potent PI3K inhibitor, inhibits OC migration toward M-CSF

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‡ The abbreviations used are: OC, osteoclast; RTK, receptor tyrosine kinase; WT, wild type; SFK, Src family kinase; PI3K, phosphatidylinositol 3-kinase; Epo, erythropoietin; EpOR, Epo receptor; RANKL, receptor activator of NFκB ligand; BMM, bone marrow-derived macrophage; Ab, antibody; ERK, extracellular signal-regulated kinase.
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and blocks bone resorption in vitro and in vivo (23, 24). Moreover, c-Src null OCs exposed to M-CSF exhibit impaired migration and a failure of cytoskeletal reorganization (25), events that contribute to their deficit in bone-resorbing activity (26). Finally, the M1 myeloid cell line, which cannot differentiate into mature osteoclasts, displays defective M-CSF-dependent differentiation into more mature macrophages when overexpressing a specific c-Fms tyrosine mutant (Phe-559) that lacks the ability to recruit Src family members, highlighting the importance of c-Fms Tyr-559 and Src family-dependent signaling (27).

The purpose of this study is to dissect the signaling pathway initiated by M-CSF that regulates the cytoskeletal rearrangement in primary macrophages and OCs, each of which expresses abundant c-Fms. To achieve this goal we transduced these cells with a chimeric receptor consisting of the extracellular domain of erythropoietin (Epo) receptor (EpoR) and the transmembrane and cytoplasmic domains of c-Fms, in which the functionally critical tyrosine residues are either unchanged or mutated to Phe, singly or in combination. We have validated this model previously in that Epo plus RANKL treatment of transduced cells expressing the WT chimerax differentiated into bone-resorbing OCs as efficiently as those exposed to RANKL and M-CSF (28).

We show here that binding of M-CSF to c-Fms initiates a series of phosphorylation events resulting in formation of a multicimeric complex required for cell motility and cytoskeletal reorganization. The complex contains SFKs c-Cbl-PI3K, and its optimal assembly following activation of the c-Fms pathway requires the simultaneous presence of three specific tyrosine residues in the cytoplasmic domain of the RTK.

MATERIALS AND METHODS

Infection, Selection, and Expansion of Bone Marrow Macrophages—Bone marrow-derived macrophages (BMMs) were isolated from long bones of C57/Bl6 mice and cultured for 2 days in α-minimal essential medium supplemented with 10% fetal bovine serum in the presence of 1:10 volume of CMG14–12 culture supernatant (CMG), equivalent to 100 ng/ml recombinant M-CSF (29). c-Cbl−/− and c-Cbl+/+ BMMs were isolated from long bones of 6/8 week-old-mice kindly provided by Dr. Roland Baron (Yale University School of Medicine, New Haven, CT). BMMs were infected with virus for 24 h in the presence of 1:10 volume of CMG and 4 μg/ml polybrene (Sigma) as described previously (30) and further cultured in the presence of 2 μg/ml puromycin and M-CSF for selection and expansion of transduced cells.

Generation and Use of OCs and BMMs—Retrovirally transduced BMMs were cultured on coverslips in α-minimal essential medium + 10% serum supplemented with 1:100 CMG alone or in the presence of 100 ng/ml RANKL for 3 days to generate pre-OCs or for 7 days to generate mature OCs.

Western Analysis—BMMs transduced with various EpoR/c-Fms chimeras or control empty vector were starved and stimulated with Epo (20 units/ml) or M-CSF (100 ng/ml). Following stimulation, cells were lysed in radioimmune precipitation buffer supplemented with protease inhibitors and sodium orthovanadate. Immunoprecipitation was performed using 200-μg protein aliquots incubated for 1 h on ice with 2 μg of primary Ab, followed by overnight incubation with protein A/G beads (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Total cell lysate Western blot analysis was performed on 40-μg protein aliquots. In brief, electrophoresis in 8% SDS-PAGE was followed by transfer to nitrocellulose, with blocking using 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were exposed overnight at 4 °C to primary antibodies, washed, and incubated with secondary goat anti-rabbit IgG horseradish peroxidase-conjugated antibody for 1 h and developed with enhanced chemiluminescence detection assay by x-rays (Pierce Chemical Co.). Films were scanned with an Epson Perfection 2400 Photo scanner and figures made in Adobe Photoshop. Some Western blots were performed using secondary antibodies labeled with IR dyes that are visualized by differential fluorescence channels (700 and 800 nm) and scanned using the Odyssey Infrared Imaging System (Li-cor). The polyclonal antibodies used were c-Fms (C-terminal, C-20), c-Cbl, and PI3K-p85 (Santa Cruz Biotechnology, Inc.). Vav3 phosphorylation was performed by immunoprecipitation with Vav3 monoclonal Ab (gift from Dr. W. Swat, Washington University, St. Louis, MO) followed by Western blot with antiphosphotyrosine Ab (clone 4G10; Upstate Biotechnology, Wall- tham, MA).

Immunofluorescence—Mature OCs expressing various EpoR/c-Fms chimeras were generated in the presence of RANKL and M-CSF prior to stimulation with M-CSF (100 ng/ml) or Epo (20 units/ml). Cells were selected with puromycin during the entire culture period. For immunofluorescence, fluorescein isothiocyanate-phalloidin was layered on OCs, fixed and permeabilized with 0.1% Triton X-100, and incubated in a humidified chamber for 45 min at 37 °C. After rinsing in phosphate-buffered saline, pH 7.6, coverslips were mounted in 20% Mowiol 4–88 (Calbiochem-Novabiochem Corp.) and viewed on a Nikon microscope (Eclipse E400). Images were acquired using an Optronics camera with Magnafire Software.

Spreading and Migration Assays—Cell spreading and chemotactic migration assays were performed using pre-OCs expressing the different EpoR/c-Fms chimeras. For both sets of experiments, BMMs were transduced with the indicated chimeras, selected in puromycin- and 10% CMG-containing media, and then cultured with RANKL and M-CSF. After 3 days, pre-OCs were lifted with trypsin, washed in PBS, counted, and used for cell spreading or migration assays. For cell spreading, cells were plated onto osteopontin-coated coverslips and adhered for 30 min in either the presence or absence of 20 units/ml Epo. Adherent cells were fixed in 4% paraformaldehyde and stained with crystal violet. For migration assay, cells were placed in transwell filters (8-μm pore size; Costar, Cambridge, MA) pre-coated with serum and allowed to migrate for 6 h toward a gradient of 20 units/ml Epo or medium alone. Cells on the lower surface of the filter were viewed at ×300 magnification in a bright field and counted. Results represent the mean value from 15 fields ± S.E. of a representative experiment.

Rac Assays—Pre-OCs expressing the indicated EpoR/c-Fms mutants were stimulated with Epo for 5 min and lysed as described previously (12). Lysates were subjected to a Rac pull-down assay using glutathione S-transferase-PAK1 beads (BD
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effectively as the WT chimera (Fig. 1). A single add-back construct recruited the p85 subunit of PI3K as residues had been replaced by Phe (Tyr-721 add-back). This null form of the chimera, in which all seven functional Tyr EpoR/c-Fms receptor in which Tyr-721 was added back to a confirmed that Tyr-721 was a p85 binding site, we generated an tion that abrogated Epo-induced p85 binding was Tyr-721. To (data not shown). As seen in Fig. 1A, 5 min of Epo treatment of cells expressing WT EpoR/c-Fms induced rapid podosome redistribution from a peripheral belt to the cell surface, yielding to numerous membrane ruffles (Fig. 2B+, insert), whereas vector alone-transduced cells failed to respond (Fig. 2C+, insert). Given that c-Fms Tyr-721 recruited PI3K, it was surprising that the Phe-721 EpoR/c-Fms responded to Epo in a manner indistinguishable from WT (Fig. 2D+, insert). Similar to OCs, macrophages expressing WT EpoR/c-Fms responded to Epo with rapid changes in actin reorganiza tion, leading to accumulation of F-actin at the cell periphery (Fig. 2F, WT, arrows). Again, mutation of the c-Fms PI3K binding site Tyr-721 to Phe resulted in Epo-induced morphological changes indistinguishable from WT (Fig. 2F, F721, arrows).

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M-CSF-induced Cytoskeletal Changes and Migration in OCs Require Functional c-Fms Tyr-559—c-Src is the other known regulator of actin dynamics in OCs (32–34). Studies involving overexpression of c-Fms in transformed fibroblasts had shown that SFKs bind to c-Fms via Tyr-559 (35). Thus, we hypothesized that this Tyr residue might be important in the M-CSF-dependent regulation of the actin cytoskeleton in OCs. We first confirmed that Tyr-559 was indeed an SFK binding site in primary macrophages (see Fig. 5A, middle panel). Next, we evaluated Epo-dependent cytoskeletal reorganization in mature OCs and BMMs expressing EpoR/c-Fms Phe-559 and found that in both cell types the mutated EpoR/c-Fms receptor failed to promote cytoskeletal reorganization in response to Epo stimulation (Fig. 2, E and F+, insert). This result was specific for mutation in Tyr-559 because all other single Tyr to Phe mutated forms of EpoR/c-Fms continued to stimulate actin remodeling (not shown).

M-CSF-induced cytoskeletal changes leading to increased motility, an event involving PI3K (23, 32, 36) and SFKs (25, 33). Thus, we determined whether movement of cells of the monocyte/macrophage lineage was mediated via c-Fms Tyr-721 and/or Tyr-559. We measured the migratory capacity of OC precursors expressing WT or mutated Tyr-721 and Tyr-559 chimeric receptors by plating them on serum-coated transwell filters and counting the number of cells migrating toward Epo-containing medium or one lacking the cytokine. Reflecting actin reorganization, Epo-directed cell motility was enhanced in cells bearing WT and Phe-721 chimeras (Fig. 3A) despite the established migratory suppressive effect of the PI3K inhibitor LY294002 (Fig. 3B and Ref. (23)). In contrast to Phe-721, Phe-559 chimera clearly decreased the chemotactic response toward Epo (Fig. 3A). Further support for the contention that binding of SFKs to c-Fms Tyr-559 was required for cytoskeletal

Transduction Laboratories). Immunoprecipitated proteins were analyzed using SDS-PAGE followed by immunoblotting against Rac1 (BD Transduction Laboratories).

RESULTS

M-CSF-dependent Cytoskeletal Organization in Osteoclasts Does Not Require Direct Recruitment of PI3K to c-Fms Tyr-721—Although PI3K mediates M-CSF-dependent cytoskeletal organization (31, 32), the c-Fms tyrosine residue/s that regulate the cytoskeletal reorganization in response to M-CSF in OCs have not been identified. To address this issue, we generated a chimeric construct comprising the external domain of the EpoR linked to the transmembrane and cytoplasmic domain of c-Fms. This construct supported osteoclast differentiation and macrophage proliferation as well as endogenous c-Fms (28). We individually mutated the seven known functional Tyr residues in the cytoplasmic domain of c-Fms to Phe and inserted a FLAG sequence at the amino terminus of the EpoR/c-Fms construct to facilitate subsequent immunoprecipitation. BMMs were transduced with WT EpoR/c-Fms receptor or with mutated forms of EpoR/c-Fms, treated with Epo, and lysed, and the lysates were immunoprecipitated with anti-FLAG Ab followed by Western blot analysis. Using an antibody that recognized the cytoplasmic domain of c-Fms and thus could quantify both the endogenous and chimeric c-Fms receptors, we confirmed that expression levels of both receptors were similar (28) (data not shown). As seen in Fig. 1A the sole Tyr to Phe mutation that abrogated Epo-induced p85 binding was Tyr-721. To confirm that Tyr-721 was a p85 binding site, we generated an EpoR/c-Fms receptor in which Tyr-721 was added back to a null form of the chimera, in which all seven functional Tyr residues had been replaced by Phe (Tyr-721 add-back). This single add-back construct recruited the p85 subunit of PI3K as effectively as the WT chimera (Fig. 1B). Furthermore, add-back Tyr-721, but not mutated Tyr-721, underwent tyrosine phos-
organization and motility was provided by the fact that PP2, the Src kinase inhibitor, completely blocked Epo-induced migration of OC precursors expressing WT EpoR/c-Fms chimeric receptor (Fig. 3B).

These observations, along with the fact that inhibition of PI3K activation by LY294002 also negatively affected cell motility (Fig. 3B) and membrane ruffling (not shown), indicated that both SFKs and PI3K mediated M-CSF-dependent migration. However, our data suggested the direct binding of PI3K to c-Fms Tyr-721 was not required for M-CSF-induced cell motility and actin dynamics, whereas intact c-Fms Tyr-559 was required for M-CSF-mediated cytoskeletal changes.

One possibility was that PI3K might govern cytoskeletal reorganization by binding to other cytoplasmic Tyr residues in the RTK. Because c-Fms Tyr-721 was the sole residue directly binding PI3K in response to M-CSF (Fig. 1), this kinase could be indirectly recruited to the RTK by forming a complex with signaling molecules involved in M-CSF signaling. Therefore, it seemed likely that SFKs and PI3K shared a common signaling pathway downstream of Tyr-559, an issue addressed below.

c-Cbl Interacts with SFKs and PI3K in a Manner Requiring c-Fms Tyr-559—c-Fms Tyr-559 is a known binding site for SFKs in macrophages (35) and in the myeloid cell line M1 (38). Following attachment of macrophages and OCs on an avb3-dependent substrate, c-Src recruits and phosphorylates c-Cbl at Tyr-731 (33), an event that could be critical for recruitment of the p85 subunit of PI3K to the heterodimer (39). Thus, we determined whether an SFK-c-Cbl complex was recruited to c-Fms in OC precursors by treating cells with M-CSF, immunoprecipitating the cell lysates with c-Cbl, and blotting for c-Fms. As seen in Fig. 4A, binding of c-Cbl to the c-Fms receptor occurred upon M-CSF stimulation. Moreover, this interaction was dramatically decreased in c-Src-deficient cells (Fig. 4A).

Given that c-Src binds Tyr-559 in c-Fms and that the SFK and c-Cbl are known to interact in other circumstances (40), we used our chimeric receptor approach to examine the role of Tyr-559 in recruitment of c-Cbl to the RTK. Cells transduced with WT chimera and those expressing Phe-559 were treated with Epo and examined for binding of c-Cbl and SFKs. Within 5 min of Epo exposure, c-Cbl and SFKs were recruited to the WT chimeric receptor, whereas the mutated Phe-559 was incapable of inducing the same interaction (Fig. 4B). Similar to WT, Phe-721 mutated receptor was capable of supporting the recruitment of c-Cbl and SFKs to c-Fms receptor following Epo stimulation. Consistent with the role of c-Cbl as a ubiquitin ligase (41), the WT and Phe-721 receptors were degraded significantly within 30 min of Epo exposure, contrasting with minimal change in the Phe-559 mutated receptor (Fig. 4B, lower panel).

The fact that Tyr-559 is a site for recruitment of an SFK-c-Cbl complex, coupled with the knowledge that c-Cbl has been reported to bind PI3K (42), suggested that c-Cbl might act as adaptor molecule mediating formation of a complex between SFK and p85. In keeping with this hypothesis, reciprocal immunoprecipitation and immunoblots demonstrated that although binding of c-Cbl to SFKs in OC precursors transduced with WT chimera was stimulated after treatment with Epo, the same interaction was not increased in cells...
expressing c-Fms Phe-559 (Fig. 5A and not shown). Cytokine-induced c-Cbl phosphorylation and binding of c-Cbl to SFKs is markedly decreased by the SFK inhibitor PP2 and significantly inhibited in cells derived from c-Src null mice (Fig. 5B). Furthermore, association of PI3K with c-Cbl paralleled phosphorylation of c-Cbl and is dependent on Tyr-559 (Fig. 5C). To further prove that c-Cbl acted as an adapter molecule mediating the interaction between SFKs and PI3K, we used c-Cbl-deficient cells stimulated with M-CSF with time and analyzed the association between SFKs and PI3K. Although binding of SFKs and PI3K occurred as early as 1 min after M-CSF stimulation and lasted for at least 30 min, this association was abrogated in c-Cbl-deficient cells (Fig. 5D).

Tyr-559 Alone Is Insufficient to Activate Cytoskeletal Reorganization—Having demonstrated that mutation of c-Fms Tyr-559 blocked M-CSF-dependent cytoskeletal reorganization and binding of c-Cbl to SFKs and PI3K, we asked whether OC precursors expressing an EpoR/c-Fms receptor in which Tyr-559 was the only functional tyrosine residue capable of transducing c-Fms signals (Tyr-559 add-back) could stimulate M-CSF-induced motility and spreading. Thus, cells expressing WT, Phe-559, or Tyr-559 add-back EpoR/c-Fms were plated on coverslips in the presence of Epo to assess spreading or in a...
Activation of Rac was detected by Rac pulldown assay. Analysis was performed with an anti-phosphotyrosine Ab (Phe-559, Phe-721, or add-back Tyr-559 chimeras with anti-Vav3 Ab after stimulation with Epo. Western blotting for c-Cbl. As expected, the presence of Tyr-559 alone, like mutated Phe-559, did not induce binding of c-Cbl to SFKs (Fig. 7B). Interestingly, mirroring its capacity to normalize Epo-induced cell motility, the add-back chimeric receptor Tyr-559/Tyr-697/Tyr-721 supported Epo-directed migration only minimally (not shown).

The fact that Tyr-559 was required for binding between SFKs and c-Cbl (Fig. 5A) prompted us to determine whether this complex formed when Tyr-559 alone or Tyr-559/Tyr-697/Tyr-721 were the sole functional Tyr residues of c-Fms. OC precursors expressing WT chimeric receptor or the add-back Tyr-559 chimera were stimulated with Epo and analyzed by immunoprecipitation of lysates from OC precursors expressing WT EpoR/c-Fms, Phe-559, Phe-721, or add-back Tyr-559 chimeras with anti-Vav3 Ab after stimulation with Epo. Western blot analysis was performed with an anti-phosphotyrosine Ab (clone 4G10), using total Vav3 levels as control. Activation of Rac was detected by Rac pulldown assay.

Because M-CSF-dependent cytoskeletal changes in macrophages (43) and OCs (44) occur via activation of Vav3 and its downstream effector Rac, we hypothesized that this signaling pathway would be dependent on functional Tyr-559. In keeping with this proposition, we found that mutation of c-Fms Tyr-559 to Phe blocked Vav3 phosphorylation and Rac activation, whereas the Phe-721 mutated receptor did not affect the activation of these two molecules (Fig. 6D). Furthermore, reflecting its inability to promote cytoskeletal reorganization, add-back Tyr-559 was not sufficient to promote Vav3 and Rac activation (Fig. 6D). In summary, these findings indicated that although Tyr-559 was required for M-CSF-dependent cytoskeletal reorganization in BMMs and OCs, this residue alone was insufficient to promote cytoskeletal reorganization.

**Tyr-559 Alone Does Not Support Formation of an SFK-c-Cbl Complex That Is Restored in the Presence of c-Fms Tyr-697 and Tyr-721**—To investigate the potential role of c-Fms Tyr residues other than Tyr-559 in M-CSF-dependent regulation of the cytoskeleton, we generated add-back mutants where Tyr-697 and Tyr-721 were co-expressed along with Tyr-559. On the basis of overexpression studies in immortalized cells, Tyr-697 was reported to be a binding site for Grb2 (45). Moreover, Tyr-697 was required to rescue the cytoskeletal reorganization by high dose M-CSF in cells lacking the integrin αvβ3 (30). Tyr-721 was chosen for its capacity to act as a binding site for PI3K. To assess the functionality of the chimeric receptors we determined their capacity to promote cell migration in response to Epo. OC precursors expressing WT, add-back Tyr-559, or add-back Tyr-559/Tyr-697/Tyr-721 EpoR/c-Fms chimeras were plated onto porous filters and allowed to migrate toward a gradient of Epo. Although Tyr-559 add-back alone did not promote OC precursor migration in response to Epo (Figs. 6B and 7A), the process was wholly rescued by Tyr-559/Tyr-697/Tyr-721 reconstitution. A combination of Tyr-559/Tyr-697 or Tyr-559/Tyr-721 add-back mutants supported Epo-directed migration only minimally (not shown).

The fact that Tyr-559 was required for binding between SFKs and c-Cbl (Fig. 5A) prompted us to determine whether this complex formed when Tyr-559 alone or Tyr-559/Tyr-697/Tyr-721 were the sole functional Tyr residues of c-Fms. OC precursors expressing WT chimeric receptor or the add-back Tyr-559 chimera were stimulated with Epo and analyzed by immunoprecipitation with a pan-SFK antibody, followed by Western blotting for c-Cbl. As expected, the presence of Tyr-559 alone, like mutated Phe-559, did not induce binding of c-Cbl to SFKs (Fig. 7B). Interestingly, mirroring its capacity to normalize Epo-induced cell motility, the add-back chimeric receptor Tyr-559/Tyr-697/Tyr-721 could fully rescue the SFK-c-Cbl complex to WT levels. In conclusion, these findings indicated that although Tyr-559 was a platform for SFK-c-Cbl/PI3K complex,
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In this study, we detailed the mechanism by which c-Fms governs M-CSF-dependent cell migration and cytoskeletal reorganization in macrophages and OCs. Our findings reveal 1) direct recruitment of PI3K to c-Fms Tyr-721, in the context of a c-Fms receptor where all the other Tyr residues are functional, is not required for M-CSF-induced cytoskeletal changes; 2) Tyr to Phe mutation of c-Fms Tyr-559 completely abrogates podosomal reorganization and OC migration in response to M-CSF via inhibiting the formation of an SFK-c-Cbl-PI3K signaling complex and the activation of Vav3 and Rac; 3) Tyr-559, although necessary, is not sufficient to mediate M-CSF signaling to the cytoskeleton; whereas 4) a combination of Tyr-559 and Tyr-697 plus Tyr-721, with all other functional Tyr residues mutated to Phe, can fully restore the formation of an SFK-c-Cbl complex and M-CSF-dependent migration.

Many cells, both transformed and primary cultures, undergo actin remodeling and cell motility following activation of specific receptor tyrosine kinases (46, 47). Cell migration and cytoskeletal reorganization are central processes common to macrophages and OCs following activation of the M-CSF receptor c-Fms. Macrophages when recruited to inflammatory sites migrate in response to M-CSF and undergo dramatic changes in the organization of their actin cytoskeleton. An analogous response is seen in resorptive OCs migrating on bone in response to a gradient of the same cytokine. Bone resorption involves a series of steps initiated by binding of the cell to the matrix via the αvβ3 integrin and formation of a polarized sealing zone that defines the area to be resorbed where protons and proteinases are secreted, followed by detachment and migration to a new site on bone in response to M-CSF, where the cycle of events is repeated (48, 49).

The upstream signals involved in actin dynamics can be initiated by RTKs, whose ligation leads to the interaction between the receptor, SFKs, and other Src homology 2 domain-containing proteins such as PI3K, c-Cbl, and Grb2 (50). SFKs are recruited to a specific phospho-Tyr residue of RTKs, contributing to cell migration induced by cytokines such as platelet-derived growth factor, stem cell factor, or epidermal growth factor (51, 52). Similar to SFKs, PI3K, c-Cbl, and Grb2 recognize domains of RTKs by ligation of a phospho-Tyr residue within the context of flanking amino acids that provide specificity for the Src homology 2 domain in each of the adaptor proteins so bound (53).

Previously, the role of individual c-Fms tyrosine residues in controlling cell function and signaling was determined by transfecting wild-type or mutated c-Fms into either fibroblasts or interleukin 3-dependent immortalized myeloid cells (27, 54, 55). However, these studies were conflicting and/or incomplete, because the receptor was overexpressed and neither cell type expresses c-Fms or can differentiate into bone-resorbing OCs, leaving questionable the signaling pathways by which M-CSF activated the receptor and its downstream signals. Furthermore, the cell lines used in earlier experiments did not exhibit the unique actin cytoskeleton of the OC. In contrast to these approaches, all our studies used exclusively primary OC precursors, which normally express high levels of c-Fms and respond to M-CSF treatment by reorganizing their actin cytoskeleton. To define the role of individual cytoplasmic Tyr residues in these cells, we developed a chimeric receptor consisting of the external domain of the EpoR fused to the transmembrane and cytoplasmic domains of c-Fms. When expressed retrovirally at physiological levels in macrophages, this chimeric receptor responded to Epo stimulation by generating bone-resorbing OCs, leaving questionable the signaling pathways by which M-CSF activated the receptor and its downstream signals. Furthermore, the cell lines used in earlier experiments did not exhibit the unique actin cytoskeleton of the OC. In contrast to these approaches, all our studies used exclusively primary OC precursors, which normally express high levels of c-Fms and respond to M-CSF treatment by reorganizing their actin cytoskeleton. To define the role of individual cytoplasmic Tyr residues in these cells, we developed a chimeric receptor consisting of the external domain of the EpoR fused to the transmembrane and cytoplasmic domains of c-Fms. When expressed retrovirally at physiological levels in macrophages, this chimeric receptor responded to Epo stimulation by generating bone-resorbing OCs when costimulated with the osteoclast-differentiating factor RANKL (28). Moreover, Epo stimulation of macrophages induces autophosphorylation of the Tyr residues within the c-Fms tail and activation of the ERK and Akt pathways with similar amplitude and timing compared with the signal activated by M-CSF binding its endogenous receptor (not shown). The similar proliferation (61) and differentiation (28) in response to either M-CSF or Epo of macrophages that contain endogenous c-Fms and express the chimeric c-Fms receptor establish the validity of our model to dissect M-CSF-dependent signaling in these cells. To ensure that the chimeric receptor is not affecting differentiation per se, OCs were generated in the presence of M-CSF and RANKL. Epo stimulation of primary macrophages or mature OCs transduced with single or combined Tyr within the cytoplasmic domain of EpoR/c-Fms permits assessment of M-CSF-dependent signals.
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We find that the c-Fms mutant Phe-559, which does not retain the ability to recruit SFKs in response to M-CSF stimulation, abolishes M-CSF-dependent membrane ruffling and cell motility. This observation is in agreement with the reported role of c-Src in M-CSF-mediated actin dynamics in OCs (25, 33). Surprisingly, despite the known role of PI3K in M-CSF-dependent signaling (32), we find that c-Fms Tyr-721, the direct binding site for PI3K, is not required for podosome redistribution or for the chemotactic response to M-CSF. The fact that a specific PI3K inhibitor, LY294002, blocks M-CSF-induced migration while the c-Fms Tyr-721 to Phe mutation continues to transmit the signal suggests that direct binding of PI3K to c-Fms is not required for signaling to the cytoskeleton. Because mutation of c-Fms Tyr-559 blocks all M-CSF-dependent actin dynamics, we hypothesized that PI3K could be indirectly recruited to a signaling complex formed at the Tyr-559 site. SFKs, when recruited to Tyr-559, do not associate with PI3K directly, because this interaction in response to M-CSF does not occur in c-Cbl-deficient cells. Thus, our data suggest that Tyr-559 is the major residue required for transduction of M-CSF-stimulated cell motility and actin dynamics in macrophages and OCs by allowing the formation of a complex between SFKs and c-Cbl and c-Cbl and PI3K.

c-Cbl is a known regulator of podosome assembly/disassembly in OCs and membrane ruffling in macrophages (56–58) and for migration in both cell types (58, 59). In the present study, we dissected the M-CSF signaling pathway leading to cell motility and clarified the role of c-Cbl in response to M-CSF using different mutants of the c-Fms receptor. We find that functional Tyr-559 regulates the binding of c-Cbl to SFKs and PI3K and the recruitment to c-Fms. In agreement with a previous study showing that in OC precursors c-Cbl binds c-Src via its Src homology 3 domain in response to integrin engagement leading to phosphorylation of c-Cbl Tyr-731 (33), we find that M-CSF-induced association between SFKs and c-Cbl is dependent on Src kinase activity and is abolished in cells expressing the c-Fms Phe-559 mutation. Furthermore, we prove that c-Cbl is required for the M-CSF-dependent association of SFKs and PI3K, because this complex fails to form in c-Cbl−/− cells.

Regulation of the OC and macrophage cytoskeleton depends on the concerted activation of the small GTPases Rho, Rac, and Cdc42, which lead to actin polymerization through N-Wiskott-Aldrich syndrome protein and the Arp2/3 complex (11, 14). M-CSF is a potent activator of Rac in OCs (12), a process dependent on the guanosine exchange factor Vav3 (44). Targeted deletion of N-Wiskott-Aldrich syndrome protein (11) or Vav3 (44), small interfering RNA-mediated down-regulation of Arp2/3 complex (14), inhibition of Rac (13), or expression of dominant-negative Rho (60) each lead to OC malfunction and defects in macrophage motility, indicating that dynamic regulation of the cytoskeleton in these cells is a fundamental requisite for their function. We find that cells expressing c-Fms Phe-559, but not Phe-721, exhibit defective Vav3 phosphorylation and Rac activation, suggesting that the SFK-c-Cbl-PI3K complex signals through Rac. These findings suggest that Tyr-559 is the sole site required for the recruitment of cytoskeletal signaling components involved in M-CSF-dependent regulation of actin dynamics. Thus, a c-Fms cytoplasmic domain in which Tyr-559 is the sole functional Tyr residue, with all others mutated to Phe (Tyr-559 add-back), should induce actin reorganization and promote motility in response to ligand binding. In contrast to these expectations, we find that Tyr-559 is required but not sufficient to initiate these signals. In fact, when Tyr-559 is the sole functional residue in the c-Fms receptor, the chimeric receptor is not capable of supporting activation of Vav3 and Rac or the formation of the SFK-c-Cbl complex, cell spreading, and motility. We can exclude the possibility that the chimeric receptor containing this single Tyr residue is not active because it promotes ERK phosphorylation (61). The fact that the SFK-c-Cbl-PI3K complex, which fails to form in cells expressing Phe-559 mutant, is also absent in cells expressing the Tyr-559 add-back suggests that other Tyr residues are required to activate or stabilize the complex. Tyr-697 is a known binding site for Grb2 and Tyr-721 recruits PI3K. Our data indicate that the presence of Tyr-697 and Tyr-721, in collaboration with Tyr-559, restores cell motility to WT levels and allows formation of the complex between SFKs and c-Cbl.

Taken together, our findings suggest a model in which formation of a signaling complex capable of stimulating remodeling of the actin cytoskeleton requires three Tyr residues in c-Fms, namely 559, 697, and 721. Tyr-559 represents the primary binding site for SFKs, which are thus activated and subsequently ligate and phosphorylate c-Cbl, which in turn recruits PI3K. The fact that the ternary complex SFK-c-Cbl-PI3K does not form in cells expressing a c-Fms receptor with the sole Tyr-559 functional residue suggests that the receptor signaling complex is stabilized by secondary binding to Tyr-697 and Tyr-721. This set of cooperative interactions enhances M-CSF-induced signaling to a level that eventuates in cytoskeletal reorganization, a key step in OC and macrophage function. Support for this signaling mechanism is provided by the fact that coordination of the epidermal growth factor receptor signaling complex is initiated by scaffolding proteins ligated to the various phospho-tyrosine residues (37). Thus, our findings are likely to be important for understanding RTK-mediated migration, a process accompanied by reorganization of the actin cytoskeleton.

In conclusion, this study analyzes the role of various c-Fms tyrosine residues in the context of M-CSF cytoskeletal rearrangement and explores the mechanisms by which SFKs, c-Cbl, and PI3K are activated and recruited to specific c-Fms residues in a biologically relevant cellular system.

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