c-Fms Tyrosine 559 Is a Major Mediator of M-CSF-induced Proliferation of Primary Macrophages*

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The molecular mechanisms by which binding of monocyte/macrophage colony-stimulating factor to its receptor c-Fms promotes replication in primary macrophages are incompletely understood, as all previous studies involved overexpression of receptor mutants in transformed cells not endogenously expressing the receptor. To address this issue we retrovirally expressed, in bone marrow-derived macrophages, a chimeric receptor containing a range of tyrosine to phenylalanine mutations in the c-Fms cytoplasmic tail. We measured incorporation of bromodeoxyuridine as a marker of proliferation and phosphorylation of ERKs, Akt, and the receptor itself. Our data indicate that tyrosine 559 is the major mediator of receptor activation and cell death, intracellular signaling, and cell proliferation and that the tyrosine residues at positions 697 and 807 play lesser roles in these events. Importantly, we find that activation of the ERK and Akt pathways is necessary but not sufficient for induction of macrophage proliferation. Using specific small molecule inhibitors we find that a combination of the Src family molecule inhibitors we find that a combination of the Src family receptor mutants in transformed cells not endogenously expressed, in bone marrow-derived macrophages, a chimeric receptor containing a range of tyrosine to phenylalanine mutations in the c-Fms cytoplasmic tail. We measured incorporation of bromodeoxyuridine as a marker of proliferation and phosphorylation of ERKs, Akt, and the receptor itself. Our data indicate that tyrosine 559 is the major mediator of receptor activation and cell death, intracellular signaling, and cell proliferation and that the tyrosine residues at positions 697 and 807 play lesser roles in these events. Importantly, we find that activation of the ERK and Akt pathways is necessary but not sufficient for induction of macrophage proliferation. Using specific small molecule inhibitors we find that a combination of the Src family kinase members (SFKs), signal transducer and activator of transcription-1, phosphoinositide 3-kinase (PI3K) and Cbl, whereas Tyr-697 and Tyr-921 recognize Grb2 (8, 10–12). A single report documents that Tyr-721 recognizes PLCy2 (13).

These experiments are compromised by the fact that c-Fms mutants were expressed in transformed cell lines, which do not express the RTK endogenously, and are thus of limited relevance regarding the physiology of M-CSF and its receptor. As an example, the effect of mutating Tyr-807 to Phe (Phe-807) on cell proliferation is controversial. This mutation severely impairs cell cycle progression in NIH3T3 and Rat-2 cells (14–16) while enhancing growth of myeloid FDC-P1 cells (17), providing a conflicting view of the impact of this residue on cell proliferation. Thus, the mechanisms by which M-CSF stimulates proliferation of authentic macrophages remain to be defined.

The MAPK cascade involving the Ras/Raf/MEK/ERK pathway is required for proliferation of macrophages in response to M-CSF (18, 19). In contrast, the signals emanating from c-Fms leading to ERK stimulation in primary cells are unclear. Given these two sets of facts, we set out to define the role of the c-Fms/ERK pathway in M-CSF-driven cell cycle progression. Our approach involves retroviral expression, in primary macrophages expressing endogenous c-Fms, of a chimeric receptor comprising the external domain of the erythropoietin receptor (EpoR), linked to the transmembrane and cytoplasmic tail of c-Fms. Treatment with M-CSF or Epo, respectively, activates the endogenous or chimeric receptor. We find that M-CSF-induced DNA replication of primary macrophages requires sig-
nals transmitted by three residues in the cytoplasmic tail of c-Fms, the dominant being Tyr-559. Activation of the ERK and Akt pathways by M-CSF is mediated by c-Fms Tyr-559 and Tyr-697, but signals required for macrophage proliferation require the additional presence of Tyr-807. Using specific small molecule inhibitors we find that a combination of the SFK, ERK, and PLC pathways mediates macrophage proliferation in response to M-CSF.

EXPERIMENTAL PROCEDURES

Reagents—Antibodies directed against the C-terminal domain of c-Fms (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Tyr-721 and -Tyr-807 of c-Fms, Akt, phospho-Akt, ERKs, and phospho-ERKs (Cell Signaling Technology, Beverly, MA), FLAG and β-actin (Sigma) were purchased commercially. Epo was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). M-CSF and RANKL were used as previously described (20). U0126 was obtained from Cell Signaling Technology and PP2 and U73122 from Calbiochem and Novachem. CMG14-12, a conditioned medium containing 1.2 μg of M-CSF/ml, was obtained and used as described (23).

DNA Constructs—Chimeric EpoR/c-Fms receptors containing individual Tyr to Phe mutations were constructed and cloned into the pMX-puro retrovirus vector as described previously (20, 21). To perform biochemical analysis of chimeric receptors, N-terminal FLAG-tagged chimeric EpoR/c-Fms receptors were constructed by adding coding sequence at the position just after the EpoR signal peptide sequence, using PCR primers (NFLAGF, 5’-ATCTTAGGCTTGTC-ATGCTTTGTAGCTGCCAGGCTGCCGAC-3’, and a vector primer NFLAGR, 5’-TTGCTAGCCCCAGCTCCC- GGACCCCAAG-3’, and an internal reverse primer). Two PCR fragments were double-digested with BamHI/AavrII or Nhel/ Xhol, respectively, and ligated into the BamHI and Xhol-digested WT chimera receptor vector. Silent nucleotide substitutions, which do not alter amino acid sequences, were introduced at positions Ala-650, His-701, Val-718, (Glu-743, Ala-744, Ser-745), and (Gly-840, Ile-841) to create new restriction sites for SphI, XbaI, SalI, HindIII, and EcoRI, respectively. Multiple combinations of Tyr to Phe mutations were established using these restriction sites.

The expression plasmids were transiently transfected into Plat-E packaging cells (22) using FuGENE 6 transfection reagent (Roche Applied Sciences). Virus was collected 48, 72, and 96 h after transfection and pooled.

Primary Macrophages and Retrovirus Infection—M-CSF-dependent bone marrow macrophages (MDMs) were cultured in α-minimal essential medium supplemented with 10% fetal bovine serum and 10% CMG14-12 culture supernatant from bone marrow cells of 6- to 9-week-old mice for 2 days as previously described (23). Then, cells were infected with virus for 24 h in the presence of 10% CMG14-12 culture supernatant and 4 μg/ml Polybrene (Sigma). Cells were cultured further in the presence of M-CSF and 2 μg/ml puromycin (Sigma) for 3 days prior to treatment with Epo and Western blot analysis. For proliferation, assay cells were cultured in the presence of either M-CSF or Epo and puromycin for 2 days, and proliferation activity was measured as described below.

MDMB Proliferation Assay—Either M-CSF- or Epo-stimulated transduced cells were labeled with 10 μM 5-bromodeoxyuridine (BrdUrd) for 2 h, and BrdUrd incorporation was measured using a Cell Proliferation Biotrak enzyme-linked immunosorbent assay (ELISA) system (Amersham Biosciences).

Cell Cycle Analysis—For cell cycle analysis, MDMs transduced with vector alone, WT, Phe-559, or Phe-807 EpoR/c-Fms were cultured in the presence of serum containing medium supplemented with 10% CMG14-12 culture supernatant and puromycin (2 μg/ml) for 2 days and then maintained further in the presence of low levels of M-CSF and puromycin for an additional 3 days, during which time cells failed to proliferate. Cells were washed with medium without serum three times and restimulated with either M-CSF (100 ng/ml) or Epo (100 ng/ml) in the presence of serum. After 24 h, cells were labeled with 10 μM BrdUrd for 1 h, and their cell cycle was characterized using the BrdUrd/7-amino actinomycin D flow kit (BD Biosciences).

Immunoprecipitation and Western Blotting—Transduced cells were starved of serum and cytokines for 6 h and restimulated with Epo for the appropriate time. Cells were lysed in a radioimmune precipitation buffer containing inhibitors as described previously (24). 200 μg of cell lysates were immunoprecipitated with anti-FLAG (Sigma) and protein A/G PLUS-agarose (Santa Cruz). Cell lysates were subjected to electrophoresis on 8% SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were then exposed to primary antibodies overnight at 4 °C, washed three times, and incubated with secondary goat anti-mouse or rabbit IgG horseradish peroxidase-conjugated antibody for 1 h. Membranes were washed extensively, and enhanced chemiluminescence detection assay was performed following the manufacturer’s directions (Pierce).

In Vitro Kinase Assay—MDMs were transduced with retrovirus coding for FLAG-tagged WT EpoR/c-Fms, all seven single Tyr/Phe point mutations, and the kinase-dead form of the receptor. Cells were selected for 2 days in puromycin, treated for 5 min with 20 units/ml Epo, lysed, and immunoprecipitated with anti-FLAG monoclonal (M2; Sigma). Following washing to remove non-specifically bound proteins, excess FLAG peptide (Sigma) was added in 50 μl of washing buffer and incubated at room temperature for 30 min to elute bound FLAG-tagged c-Fms. Equal aliquots were removed, added directly to kinase activity ELISA plates containing covalently bound pan-RTK substrate E4YD (catalog number 539701; Calbiochem), and the assay was performed as described in the manufacturer’s instructions. The results from triplicate wells were used to calculate mean and S.D. for each receptor mutant.

RESULTS

Endogenous c-Fms and a Chimeric Receptor Stimulate Macrophage Proliferation Equivalently—M-CSF is required for proliferation of macrophages, but the signals by which the cytokine stimulates cell division in authentic target cells are unknown. To address this issue we used MDMs, which express c-Fms endogenously (10, 23, 25). We retrovirally transduced these cells with a chimera containing the extracellular domain of the erythropoietin
c-Fms Signaling in Primary Macrophages

**A** Position of seven Tyr residues within wild type (WT) EpoR/c-Fms cytoplasmic domain. Denotes autophosphorylated tyrosine residue, and closed boxes denote Tyr residues that have been mutated to Phe. 7F represents all seven Tyr to Phe mutations. **B** Proliferation of macrophage transductants in response to M-CSF or Epo. MDBMs were infected with retrovirus containing WT EpoR/c-Fms chimeric receptor. After 1 day, virus-infected cells (1 × 10^5) were cultured in the presence of M-CSF or Epo plus puromycin (2 μg/ml) for 2 days. 2 h of incorporation of BrdUrd (final concentration 10 μM) into DNA was measured by a commercial ELISA kit. Data are expressed as mean OD ± S.D. in M-CSF versus Epo-treated cultures (n = 5). **C** Expression levels of endogenous c-Fms and EpoR/c-Fms chimeric receptors. Cells lysates were subjected to Western blot analysis with antibodies to the cytoplasmic tail of c-Fms that recognize endogenous c-Fms (160 and 140 kDa) and chimeric receptors (WT, kinase-dead (KD), and 7F) (90 kDa). The level of β-actin is a loading control.

receptor and the transmembrane and intracellular domains of mouse c-Fms (EpoR/c-Fms). To facilitate our studies, we inserted a FLAG tag (DYKDDDDK) between the signal sequence and the first amino acid of EpoR. The virus also confers puromycin resistance on transduced cells.

We first compared the proliferative capacity of activated WT chimeric receptor, whose c-Fms cytoplasmic tail contains all seven Tyr residues known to be phosphorylated to that of endogenous c-Fms (Fig. 1A). Cells were infected with WT virus for 24 h and then cultured for 2 days in the presence of puromycin and increasing doses of either M-CSF or Epo, after which proliferation was determined by incorporation of BrdUrd into replicating DNA for 2 h. Fig. 1B shows that activation of either endogenous c-Fms or the WT chimeric receptor resulted in indistinguishable, dose-dependent rates of proliferation. Neither kinase-dead K614M EpoR/c-Fms nor the chimera in which all seven Tyr residues are mutated to phenylalanine (Phe) (Fig. 1A, 7F) stimulated incorporation of BrdUrd or led to autophosphorylation of c-Fms (data not shown). Thus, both the kinase activity of c-Fms and some combination of Tyr residues are required for MDBM proliferation. In selected studies, cells were transduced with virus containing no EpoR/c-Fms insert and grown for 2 days in puromycin plus M-CSF or Epo. As expected, the only cells that survived were those exposed to M-CSF, which activated endogenous c-Fms (data not shown).

To confirm that our results do not reflect aberrant expression levels of the various chimeras, we quantitated endogenous and chimeric receptors using an antibody that recognizes the cytoplasmic tail of c-Fms. In each circumstance expression of c-Fms and EpoR/c-Fms was comparable (Fig. 1C), a finding that also extends to all other chimeric receptors reported in this study (data not shown).

**c-Fms Tyr-559, Tyr-697, and Tyr-807 Each Contribute to Macrophage Proliferation**—Having established that the proliferative capacity of EpoR/c-Fms mirrors that of the endogenous receptor, we determined the ability of each c-Fms cytoplasmic tail Tyr residue to transmit critical mitogenic signals in primary macrophages. Using transformed cells, others find that seven of the twenty Tyr residues in the C-terminal tail of c-Fms bind Src homology 2-containing proteins when phosphorylated, leading to downstream signal amplification (8, 10). We therefore asked which Tyr residues are critical for proliferation of primary macrophages. To address this question, MDBMs were infected with viruses encoding chimeric receptors, each containing an individual Tyr to Phe mutation. The cells were then cultured in the presence of puromycin and M-CSF. After 2 days of selection, proliferation was again measured by brief BrdUrd incorporation in response to Epo or M-CSF. As seen in Fig. 2, mutating c-Fms Tyr-559 or Tyr-807 to Phe decreased the proliferative capacity of MDBMs by ∼80%. Mutation of Tyr-697 also significantly impaired macrophage proliferation, but the impact of this residue was substantially less than that of Tyr-559 or Tyr-807. All other single mutations were without effect. To confirm these data, we determined cell cycle progression rates of macrophages expressing WT, Phe-559, and Phe-807 EpoR/c-Fms. In this experiment, MDBMs bearing empty vector or the three forms of EpoR/c-Fms were synchronized by culture for 3 days with minimal amounts of M-CSF, stimulated to reenter the cell cycle with M-CSF or Epo, and analyzed by combined BrdUrd and 7-amino actinomycin D labeling of DNA as described under “Experimental Procedures.” As expected, the percentages of cell cycle progression in response to M-CSF exposure were similar (Fig. 3). Likewise, cells bearing empty vector and exposed to Epo were essentially S-phase negative, indicating that they are unable to transit through G1 phase and into S phase. While S-phase transition of Epo-treated WT EpoR/c-Fms transductants approximated that of the same cells exposed to M-CSF, the process was blunted in cells expressing the Phe-559 or Phe-807 mutations. Thus, these data also reveal that Tyr-559 and Tyr-807 mediate signals that suppress apoptosis.
c-Fms Tyr-559, Tyr-697, and Tyr-807 Are Essential for Macrophage Growth—Given that c-Fms Tyr-559, Tyr-697, and Tyr-807 stimulate cell cycle progression, mutation of these same amino acids should also decrease cell number in longer term culture. To test this hypothesis, cells transduced with EpoR/c-Fms bearing Phe-559, Phe-697, or Phe-807 were treated with optimal levels of Epo and counted after 2, 4, and 6 days. To compare the consequences of activating the endogenous or chimeric c-Fms receptors, the same cells were also exposed to an optimal concentration of M-CSF.

Macrophages expressing EpoR/c-Fms containing any of the Tyr to Phe mutants remained responsive to M-CSF, as exhibited by similar increases in total viable cells over time (Fig. 4). In contrast, when stimulated with Epo for several days, cell number paralleled the degree to which proliferation is blunted (Fig. 2).

c-Fms Tyr-721 and Tyr-921, but Not Tyr-706, Combine with Tyr-559, Tyr-697, or Tyr-807 to Modulate Macrophage Proliferation—In addition to amino acids Tyr-559, Tyr-697, and Tyr-807, c-Fms contains several other Tyr residues implicated in regulating c-Fms signal transduction in transformed cells (8, 10). Although we found individual mutants of these additional residues failed to impact macrophage proliferation (Fig. 2), the possibility existed that they function in the context of Tyr-559, Tyr-697, or Tyr-807 to regulate macrophage growth. To determine the role of these additional Tyr residues in authentic target cells, we expressed a series of multiple EpoR/c-Fms Tyr to Phe mutants in MDBMs (Fig. 5A) and analyzed their impact on proliferation.

ERKs are required for macrophage proliferation (19, 26), and Grb2 is a key adaptor protein in the RTK stimulation of the ERK pathway (27). Consistent with these facts, the double mutant F697/F921 completely blocked Grb2/c-Fms association in immortalized cells (28), suggesting that cells bearing the construct would be minimally responsive to Epo. Although substantially reduced, significant proliferation is still observed in...
MDBMs expressing the double mutant (Fig. 5B). Thus, the findings reported in immortalized cells regarding the essential role of c-Fms Tyr-697 and Tyr-721 do not reflect accurately the situation in primary macrophages. Tyr-697, Tyr-706, and Tyr-721 reside in the c-Fms kinase insert. Mutation of each to Phe blocks proliferation of Rat-2 fibroblasts (16) but slightly enhances the same process in the myeloid-derived cell line FDC-P1 (17), again underscoring the contradictory role of these residues in regulating proliferation in different immortalized and transformed cell types. In contrast to these conflicting results in cell lines, primary macrophages bearing the triple EpoR/c-Fms mutant continued to replicate DNA as 50% as effectively as WT cells (Fig. 5B). As noted previously (Fig. 2), the proliferative capacity of cells expressing the EpoR/c-Fms single mutants Phe-559 or Phe-807 was reduced by nearly 80% compared with that of endogenous or WT chimaera receptors. Moreover, macrophages expressing the Phe mutants of these two Tyr residues in combination were essentially non-proliferative in response to Epo (Fig. 5B), again providing evidence that under physiological stimulation Tyr-559 and Tyr-807 are critical mediators of mitogenic macrophage signaling. Of interest in light of their failure to impact proliferation when expressed individually, Phe-697, Phe-721, or Phe-921 also completely ablated MDBM proliferation in combination with Phe-559. However, Phe-706 did not alter the effect of Phe-559, indicating specificity in the additive effects of Tyr to Phe mutants in combination with Phe-559. Consistent with these findings, triple or quadruple mutants containing combinations of Phe-559, Phe-697, Phe-721, Phe-807, or Phe-921 each failed to stimulate cell proliferation. Taken as a whole, the data in Figs. 1–5 indicate that (a) the original hypothesis that Tyr-697 and Tyr-721 are the sole transducers of c-Fms signaling is incorrect, (b) Tyr-559, Tyr-697, and Tyr-807 mediate M-CSF-dependent macrophage proliferation, with Tyr-559 the single most important residue, and (c) Tyr-559 and Tyr-807 transmit signals that suppress macrophage apoptosis. The detailed nature of these downstream pathways is beyond the scope of the present study.

A Subset of c-Fms Cytoplasmic Tail Tyr Residues Is Sufficient to Transmit Proliferative Signals—The experiments detailed thus far have involved chimeric receptors in which the c-Fms cytoplasmic tail carries one or several Tyr to Phe point mutations, with the remaining Tyr residues unchanged. Although this approach allows us to deduce the consequence of eliminating a given residue, it fails to provide direct evidence of the contribution of individual amino acids to c-Fms function. To address this issue, we used retroviral transduction to systematically add back individual Tyr residues to inactive EpoR/c-Fms (Fig. 6A) in which all seven Tyr residues had been mutated to Phe (Fig. 1A, 7F). In addition to the three critical residues, Tyr-559, Tyr-697, and Tyr-807, we included Tyr-921, as we have established its importance in M-CSF-stimulated preosteoclast cytoskeletal organization (46).

As shown in Fig. 6B, Tyr-559 was essential for detectable EpoR/c-Fms-mediated macrophage proliferation. Whereas Tyr-559 was ineffective alone, chimeric receptors containing any combination of Tyr-559 with Tyr-697, Tyr-721, or Tyr-807 promoted limited cell proliferation. Consistent with such addi-
tive effects, reconstitution of EpoR/c-Fms 7F with Tyr-559/Tyr-697/Tyr-721 or Tyr-559/Tyr-697/Tyr-807 significantly restored S-phase entry. Notably, the latter combination restored cell cycle progression to levels similar to that of the WT chimeric receptor. Confirming our earlier findings demonstrating dissociation between proliferation and ERK and Akt activation, there was little correlation between the ability of any given Tyr residue add back to induce these signaling molecules, on the one hand, and BrdUrd incorporation on the other (compare, for example, lanes 1, 2, 6, 7, 8, 12, and 13 in Fig. 6B with lanes 2, 4, 12, 14, 16, 24, or 26 in Fig. 6C). Thus, maximal c-Fms signaling into the cell cycle machinery requires only three of the seven Tyr residues in the cytoplasmic tail of the receptor. Furthermore, activation of the ERK and Akt pathways is not itself sufficient to stimulate completely the proliferative capacity of primary macrophages.

c-Fms Tyr-559, Tyr-697, and Tyr-807 Mediate Different Signals—To determine whether distinct intracellular signals emanate from each of the three critical c-Fms Tyr residues, we once again used M-CSF- and Epo-treated primary macrophages transduced with EpoR/c-Fms mutants. Thus, MDBMs transduced with WT EpoR/c-Fms were serum starved for 6 h and restimulated with either M-CSF or Epo for various times to activate endogenous c-Fms or chimeric receptor, respectively.

As our readout we focused on the ERK and Akt pathways that are activated in macrophages by M-CSF (29, 30). Fig. 7 shows that the patterns of phosphorylation of Akt and ERKs in cells treated with either M-CSF or Epo were identical in both time course and intensity.

Having established that endogenous c-Fms and transduced WT chimeric receptor activate the same downstream effector molecules, we turned to the role of individual Tyr residues in the activation of ERKs and Akt. To this end we transduced MDBMs with virus expressing one of four chimeric EpoR/c-Fms receptors, WT, Phe-559, Phe-697, or Phe-807. Following expansion with M-CSF under puromycin selection, cells were grown for an additional 3 days, deprived of serum for 6 h, and then treated with Epo or M-CSF with time. Lysates were prepared, and the extent of ERK and Akt phosphorylation was determined by Western blot analysis.

Consistent with its key role in macrophage proliferation, Tyr-559, when mutated to Phe, markedly dampened c-Fms-mediated Akt and ERK activation (Fig. 8). In contrast, cells expressing the Phe-807 form of the chimera phosphorylated Akt normally in response to Epo and exhibited only mildly impaired ERK activation. Of interest, the Phe-697 mutant receptor, which exhibits a limited impact on proliferation of primary macrophages, failed to alter Akt phosphorylation but substan-
c-Fms Signaling in Primary Macrophages

The data indicate that specific Tyr residues in the cytoplasmic tail of c-Fms contribute differentially to the induction of two major intracellular signaling pathways considered central to regulation of cell cycle progression.

To determine whether chimera-derived signals interfere with those emanating from the endogenous receptor, cells expressing either Phe-559 or Phe-807 (the two single mutants having the greatest impact on both proliferation and signaling) were treated simultaneously with M-CSF and Epo. Activation of the mutant forms of EpoR/c-Fms failed to impair the capacity of the endogenous receptor to transmit proliferative signals in response to M-CSF (data not shown).

SFK, PI3K, and PLC Pathways Lead to Macrophage Proliferation through ERKs—Because mutations in the cytoplasmic tail of c-Fms that suppress ERK and Akt signaling fail to completely inhibit proliferation, we performed studies to identify the additional signal(s) that may play such a role. In addition to the ERK and Akt pathways we focused our attention on PLC signaling for a combination of two reasons. First, Tyr-721 is a binding site for PLC (13), and second, it has been reported that this pathway contributes to proliferation in other cell types (19, 31–33).

To examine whether combined blockade of these three signaling pathways blunts macrophage proliferation, we used specific kinase inhibitors and assessed BrdUrd incorporation and ERK activation. We first examined the dose dependence of the SFK inhibitor PP2, the PI3K inhibitor LY294002, and the PLC inhibitor U73122, using a BrdUrd incorporation assay into MDBMs (data not shown). Although U73122 alone did not inhibit proliferation, it partially dampened the process in combination with PP2. Moreover, LY294002 blunted cell division in the presence of both PLC and SFK inhibitors (Fig. 9A). Next we analyzed the relationship between proliferation capacity and ERK activation. Fig. 9B shows that the blocking of SFK, PI3K, and PLC strongly reduced ERK activation in a dose-dependent manner, even though the PLC inhibitor alone did not influence ERK phosphorylation. Finally, we examined ERK activation by add-back Tyr mutants in the presence of combinations of the three kinase inhibitors (Fig. 9C). Add back of Tyr-559 alone stimulated activated ERKs even in the presence of the PI3K inhibitor LY294002, suggesting that SFKs activate at least two pathways. Given this observation, it is unclear why ERK activation via Tyr-559/807 was totally inhibited by LY294002. Signaling to ERKS through either c-Fms Tyr-559/697/721, which represents the kinase loop mutants, or, alternatively, the triple add-back 807/Tyr-559/697/807, was unaltered by either SFK or PI3K inhibitors alone, again reflecting the existence of separate signals downstream of Tyr-559. Consistent with this hypothesis, a combination of PP2 and LY294002 resulted in complete suppression of ERK activation. In all circumstances the use of all three inhibitors ablated ERK phosphorylation, as did the use of U0126, a potent inactivator of MEK, the immediate upstream kinase for ERKs (10). In summary, our data indicate that the SFK, PI3K, and PLC pathways modulate macrophage proliferation by regulating ERK activation.

Site-specific Autophosphorylation of c-Fms Is Regulated by Different Cytoplasmic Tail Tyrosine Residues—With the knowledge that Tyr residues 559, 697, and 807 in c-Fms are sufficient to transduce the signals required for proliferation of primary macrophages, we turned to the first signal in the c-Fms cascade, namely rapid autophosphorylation of the receptor itself. MDBMs bearing the three key EpoR/c-Fms Tyr mutants, Phe-559, Phe-697, and Phe-807, were starved of serum and cytokine for 6 h and then stimulated with Epo or vehicle for 5 min. Lysates were prepared and chimeric receptors immunoprecipitated using anti-FLAG antibody. Western blotting with a phosphotyrosine-specific antibody was used to determine the extent of receptor autophosphorylation, manifest as a band at 90 kDa, the predicted size of the EpoR/c-Fms chimera. To normalize the findings, we established the level of chimeric receptor in each lane with the same anti-FLAG antibody. As internal control, we treated the same transfected MDBMs with M-CSF and assessed phosphorylation of endogenous c-Fms, which appears at 160 kDa.

As expected, the most prominent phosphorylated species in native MDBMs acutely treated with M-CSF was the receptor itself (Fig. 10A, top panel, and Ref. (2)). Similarly, Epo strongly stimulated phosphorylation of both the WT chimera and the Phe-697 mutant. In contrast, phosphorylation of the chimeric
receptor containing the key functional mutation Phe-559 was markedly impaired. Despite the fact that EpoR/c-Fms Phe-807 arrests cell division, MDBMs bearing the mutant actually exhibited enhanced chimera phosphorylation both in the basal state and after Epo stimulation. This phenomenon does not reflect increased receptor expression or degradation, because anti-FLAG Western analysis demonstrates that the amounts of WT and Phe-807 receptors were similar in the absence of and following Epo treatment (Fig. 10A, lower panel). Consistent with the demonstrated correlation between c-Fms phosphorylation and rapid degradation (2), the relative abundance of EpoR/c-Fms Phe-559, which is poorly phosphorylated in response to Epo, remained unaltered. In contrast, the reduced quantity of chimeric receptor Tyr-807 and Tyr-697 reflected their autoactivation and subsequent degradation.

To confirm our data that various EpoR/c-Fms mutants were expressed and functional in intact cells, we performed in vitro kinase assays. In this circumstance, we expressed WT and kinase-dead chimeric receptor plus versions containing individual Tyr/Phe cytoplasmic tail mutations. Following treatment of cells with Epo, lysis, and immunoprecipitation with anti-FLAG antibody, beads were washed to remove nonspecifically bound proteins. The bound kinase was eluted with excess FLAG peptide, and equal aliquots (determined by separate

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c-Fms Signaling in Primary Macrophages

FLAG Western blot analysis were used in triplicate in a commercially available tyrosine kinase assay (K-LISA kit; Calbiochem). At a fixed time, shown in preliminary studies to provide linear data, the extent of substrate phosphorylation was determined by a modified ELISA. The kinase activity of the mutants examined paralleled the extent of phosphorylation of the mutants in Fig. 8A (not shown).

**c-Fms Tyr-559 and Tyr-697 Regulate Autophosphorylation of Tyr-807**—c-Fms Tyr-807 is a principal autophosphorylation target (10), and we asked whether this event is mediated by Tyr-559 and/or Tyr-697. Using a c-Fms phospho-Tyr-807-specific antibody, we found that chimeric receptors expressing the Phe-559 and Phe-697 mutants, alone or in combination, block Epo-induced Tyr-807 phosphorylation (Fig. 10B, left panel). In contrast, Phe mutations of Tyr-706, Tyr-721, Tyr-921, or Tyr-974, either alone or in combination, are without significant effect (data not shown). To confirm the importance of Tyr-559 and Tyr-697 in phosphorylating Tyr-807, we added back combinations of these three residues to the inactive 7F EpoR/c-Fms receptor (Fig. 1A). When Tyr-807 was reconstituted alone or in conjunction with Tyr-559 or Tyr-697 it failed to undergo Epo-induced phosphorylation. However, when Tyr-807 was added back in the context of both Tyr-559 and Tyr-697, receptor phosphorylation was indistinguishable from that occurring in the WT chimera (Fig. 10B, right panel). Thus, c-Fms Tyr-807, although key for receptor signaling, does not direct its own phosphorylation, an event in which Tyr-559 and Tyr-697 play a central role.

To determine specificity of Tyr-807 phosphorylation, we performed similar experiments using an antibody recognizing phosphorylated Tyr-721. In this circumstance, mutation of Tyr-807 to Phe had little impact on EpoR/c-Fms Tyr-721 autophosphorylation, whereas the presence of Phe-559 or Phe-697 partially suppressed this modification (Fig. 10C, left panel). Furthermore, the presence of only Tyr-721 in the EpoR/c-Fms chimeric receptor enabled significant, if not optimal, autophosphorylation (Fig 10C, right panel). Importantly, the presence of only Tyr-697 and Tyr-721 resulted in Epo-induced Tyr-721 phosphorylation indistinguishable from that occurring in WT chimeric receptor. Taken together, the findings in Fig. 10, B and C, reveal that whereas the single residue Tyr-697 in c-Fms is sufficient to induce autophosphorylation at Tyr-721, a combination of Tyr-559 and Tyr-697 is required to perform the analogous reaction at Tyr-807.

**DISCUSSION**

The human kinome includes over 500 members, of which 58 are RTKs (34), each of which contains an extracellular domain that binds a specific cytokine with high specificity, a single transmembrane region of ~25 amino acids, and a cytoplasmic tail of variable length. These proteins reside in the plasma membrane as monomers that dimerize and become activated following ligand binding. Structural analysis of the kinase domain of a number of RTKs reveals that the general topology of this region is conserved over all such enzymes (35).

Phosphorylation of cytoplasmic tail tyrosines within RTKs typically prompts specific docking and signaling protein binding to the modified site via Src homology 2 or PTB domains (9). Because a number of the bound molecules are themselves kinases or recruit other kinases to the RTK, the membrane-associated initial activation leads to a wave of intracellular signals. c-Fms itself has twenty Tyr residues in its cytoplasmic tail, of which seven, located at positions 559, 697, 706, 721, 807, 921, and 974 (murine numbering), are functional in various transformed cell lines.

Osteoclasts, the exclusive bone-resorbing cells, are generated from MDMBs by a process requiring only receptor activator of NF-κB and M-CSF. The former molecule, a member of the tumor necrosis factor superfamily, is the major regulator of osteoclast formation and function. On the other hand, M-CSF, binding to its cognate receptor c-Fms, is responsible for the survival and proliferation of MDMBs. Although it is unclear how proliferation signals correlate with those that transmit inhibition of apoptosis, we find that both Tyr-559 and Tyr-807 are essential for osteoclast survival. Whereas proliferating cells require mitogenic signals, osteoclasts do not proliferate and therefore signals required for cell survival can be characterized. Analysis of the pathways downstream of c-Fms that control cell death are a future goal.

The aim of this study was to define the mechanisms by which M-CSF modulates the proliferative capacity of MDMBs, which are osteoclast precursors. All experiments were performed with primary macrophages, which express high levels of c-Fms and cannot be transfected. To determine the role of individual Tyr residues in cell proliferation and signaling of authentic osteoclast precursors, we retrovirally transduced a receptor containing the external domain of the EpoR plus the transmembrane region and cytoplasmic tail of murine c-Fms into these primary cells. The fact that activation of this chimera yields macrophage proliferation and signals identical to that transmitted by native c-Fms validates its utilization as a determinant of the *bona fide* function of intracellular components of the M-CSF receptor. Thus, we were positioned to determine which c-Fms cytoplasmic domain Tyr residues mediate its biological effects. In fact, we find that c-Fms Tyr-697, Tyr-807, and especially Tyr-559 regulate short term proliferation and longer term growth of MDMBs. Butressing the hypothesis that a subset of Tyr residues in the c-Fms tail control MDMB function, proliferation and signaling of macrophages expressing EpoR/c-Fms containing only these three functional tyrosine residues is indistinguishable from activation of the endogenous M-CSF receptor.

Tyr-559, the major residue controlling M-CSF signaling, is the c-Fms binding site for SFKs in NIH3T3 fibroblasts (8, 10, 36, 37), and we have established the same observation in primary macrophages (46). Although controversial because the study was performed in transformed cells, Src family members recruited to Tyr-559 play an important role in subsequent proliferation (37). Three groups have reported on the role of Tyr residues that bind SFKs in mediating proliferation of myeloid cells. Although Rohde et al. (38) find that 32D cells expressing c-Fms Phe-559 are hyperproliferative, others (39) find no statistical change in the same parameter when the identical mutant receptor is expressed in M1 cells, which also do not express c-Fms endogenously. Finally, Tan et al. (40) demonstrate that in a third myeloid line, G1E-ER2, absence of Tyr-567
and/or Tyr-569, the SFK binding sites in the closely related RTK c-kit, leads to a failure of cell growth. These conflicting results, coupled with earlier findings in fibroblastic cells, themselves discordant (16, 37, 41–43), underscore the need to examine receptor signaling in the appropriate cellular context.

Taken together, these contradictory observations prompted us to establish which cytoplasmic residues are central to the activation of the c-Fms effector molecules Akt and ERKs, each of which participates in macrophage replication. Our data indicate that macrophage proliferative signals emanating from c-Fms Tyr-559 are mediated via the ERK and PI3K/Akt pathways but that these signals alone are not sufficient to drive cell proliferation of MDBMs. Using combinations of inhibitors of intracellular signal transduction, we demonstrated that the SFK, PI3K, and PLC pathways together regulate macrophage proliferation by targeting MAPKs. Confirming results, coupled with earlier findings in fibroblastic cells, thematically inactive receptor restores partially the capacity of G1E-ER2 cells to undergo proliferation (40, 45), observations that again are at odds with ours. When considered with the analysis of individual Tyr to Phe point mutants in c-Fms in transformed cell lines (38, 39), these findings re-emphasize the fact that studies in primary cells provide unique insights into signal transduction pathways.

Autophosphorylation of c-Fms is the first signaling event in M-CSF-mediated macrophage proliferation. Having established an essential role for c-Fms Tyr-559, Tyr-807, and Tyr-697 in MDBM replication and survival, we asked whether these residues also regulate receptor autophosphorylation. Again, the experiments involving point mutations of Tyr to Phe were complemented by add-back studies in which only specific Tyr residues were present in the cytoplasmic tail of c-Fms. The results indicate that Tyr-559 is essential for robust phosphorylation of both c-Fms and, specifically, Tyr-807 in its cytoplasmic tail. However, Tyr-559 has a lesser effect on phosphorylation of Tyr-721, which is regulated mainly by Tyr-697. The latter residue is not necessary for robust phosphorylation of the receptor or Tyr-807. Finally, although Tyr-807 cannot initiate total c-Fms phosphorylation when expressed alone or with either Tyr-559 or Tyr-697, the singly phosphorylated species leads to activation of Tyr-721. Thus, individual c-Fms tyrosine residues regulate global and site-specific receptor autophosphorylation to varying and opposite degrees, indicating a physiologically complex mechanism of activation.

In summary, although our results confirm selected aspects of the existing paradigm on the role of individual Tyr residues in c-Fms function, they contradict others and overall provide the first coherent functional model of this RTK in proliferation of primary cells that express the receptor endogenously. This strategy stands in contrast to previous approaches that were based on transient overexpression of c-Fms in fibroblastic cell lines or immortalized and/or transformed myeloid cells that do not endogenously express the receptor and cannot differentiate into authentic osteoclasts.

A model integrating our findings is shown in Fig. 11. Tyr-807, which is located in the activation loop of c-Fms, is not necessary for overall receptor phosphorylation or signal transduction but is required for proliferation. Based on structural evidence (35) and the fact that no protein has been identified as binding this residue in c-Fms (10), it is probable that phosphorylation of Tyr-807 alters receptor conformation (8), allowing phosphorylation of and subsequent recruitment of the appropriate signaling and/or adaptor molecules to Tyr-559. Because mutation or add-back of Tyr-721 has a limited influence on any aspect of c-Fms biology, its phosphorylation may reflect receptor autoactivation and/or change in conformation. The fact that Tyr-697 only partially controls phosphorylation of Tyr-807, and
c-Fms Signaling in Primary Macrophages

**FIGURE 11. Model of c-Fms signal transduction.** Cytokine-mediated dimerization of c-Fms leads to autophosphorylation on Tyr-807, a process controlled largely by the presence of tyrosine residues at positions 559, 697, and 721 (curved arrows; arrow thickness reflects relative role of tyrosine residues). Modification of Tyr-807 results in conformational changes that are important for c-Fms function (dashed arrow). Signals emanating from Tyr-559, Tyr-697, and Tyr-721 activate ERKs and hence proliferation via a combination of SFK, PI3K-Akt, and PLC pathways.

hence all downstream events, explains its relatively modest impact on signaling and cell proliferation.

Our unique system of c-Fms signaling reconstitution has allowed us to determine unequivocally the relative impact of individual and grouped c-Fms tyrosine residues on primary macrophage biology. Clearly, the three critical residues, Tyr-559, Tyr-697, and Tyr-807, are decisive mediators of c-Fms signal transduction. In fact, our data indicate that these three residues are capable of fully stimulating macrophage proliferation. Moreover, we reported previously that the Tyr-559 and Tyr-807 residues regulate the c-Fms-driven component of osteoclast differentiation (20). It remains to be established how these residues integrate diverse signaling pathways into various components of the cell cycle to regulate c-Fms-mediated proliferation.

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