A CSF-1 Receptor Phosphotyrosine 559 Signaling Pathway Regulates Receptor Ubiquitination and Tyrosine Phosphorylation

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Receptor tyrosine kinase (RTK) activation involves ligand-induced receptor dimerization and transphosphorylation on tyrosine residues. Colony-stimulating factor-1 (CSF-1)-induced CSF-1 receptor (CSF-1R) tyrosine phosphorylation and ubiquitination were studied in mouse macrophages. Phosphorylation of CSF-1R Tyr-559, required for the binding of Src family kinases (SFKs), was both necessary and sufficient for these responses and for c-Cbl tyrosine phosphorylation and all three responses were inhibited by SFK inhibitors. In c-Cbl-deficient macrophages, CSF-1R ubiquitination and tyrosine phosphorylation were substantially inhibited. Reconstitution with wild-type, but not ubiquitin ligase-defective C381A c-Cbl rescued these responses, while expression of C381A c-Cbl in wild-type macrophages suppressed them. Analysis of site-directed mutations in the CSF-1R further suggests that activated c-Cbl-mediated CSF-1R ubiquitination is required for a conformational change in the major kinase domain that allows amplification of receptor tyrosine phosphorylation and full receptor activation. Thus, the results indicate that CSF-1-mediated receptor dimerization leads to a Tyr-559/SFK/c-Cbl pathway resulting in receptor ubiquitination that permits full receptor tyrosine phosphorylation of this class III RTK in macrophages.

Growth factors bind their cognate receptor tyrosine kinases (RTKs) and activate various signaling pathways that mediate a broad range of biological responses (1, 2). Colony-stimulating factor 1 (CSF-1) regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells (3). Effects of CSF-1 on these target cells are mediated by the CSF-1 receptor (CSF-1R), a ~165 kDa glycoprotein that is encoded by the c-fms proto-oncogene (4). The CSF-1R belongs to the class III RTK subfamily, which includes the platelet-derived growth factor receptor α/β (PDGFαR/β), the stem cell factor receptor (SCFR) encoded by the protooncogene c-kit, and Flt3/Flik2 (2). This family is distinguished by five immunoglobulin (Ig)-like loops (D1-D5) in the extracellular domain, a single transmembrane domain, a cytoplasmic juxtamembrane domain (JMD), a split cytoplasmic kinase domain comprising an ATP-binding domain, a kinase insert sequence and a major kinase domain and a C-terminal tail. The D1-D3 domains of the CSF-1R constitute the ligand-binding region (5–7), and the D4 and D5 domains mediate receptor-receptor interaction that is indispensable for the high-affinity ligand binding and receptor activation (8, 9).

Ligand binding to RTKs is believed to induce conformational alterations of the extracellular domain and subsequent receptor dimerization stabilizes the interactions between adjacent cytoplasmic domains of the receptors, resulting in phosphorylation of cytoplasmic tyrosine residues (reviewed in Refs. 10, 11). These phosphoryrosines either relieve the autoinhibition of kinase activity by the JMD and the activation loop (12), or serve as docking sites for downstream signaling proteins containing Src-homology 2 (SH2) or phosphotyrosine-binding (PTB) domains (reviewed in Ref. 13). Previous studies of the CSF-1R have identified eight cytoplasmic domains that are phosphorylated in the activated CSF-1R (reviewed in Refs. 3, 14, 15). Relevant to the present work is the JMD Tyr-559, a switch residue that functions in kinase regulation (16–18). Phosphorylation of Tyr-559 creates the binding site for the Src family kinases (SFKs) and plays an important role in the association of E3 ubiquitin ligase c-Cbl with the receptor (16). The magnitude and duration of signaling through activated RTKs is tightly regulated by ubiquitination-dependent downregulation. The E3 ubiquitin ligase, c-Cbl (20, 21), recognizes phosphorylated tyrosine residues present on activated RTKs via the tyrosine kinase binding (TKB) module. It recruits ubiquitin-conjugating enzymes or ubiquitin-carrier enzymes by the RING finger domain and catalyzes RTK ubiquitination, which leads to receptor internalization and degradation (reviewed in Ref. 22). c-Cbl is primarily expressed in hematopoietic cells (23, 24) and in macrophages, c-Cbl-deficiency results in compromised CSF-1R ubiquitination, endocytosis, and degradation (25). c-Cbl either binds CSF-1R directly (26), or through other molecules such as Grb2, PI3K (27), or Src-like adaptor protein 2 (SLAP-2), a hematopoietic adaptor protein which interacts with the CSF-1R and c-Cbl and plays a role in c-Cbl-dependent down-regulation of CSF-1R signaling (28). On the other hand, as a multidomain adaptor protein, c-Cbl also acts positively in integrin-engaged spreading of macro-

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S8.

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2 The abbreviations used are: RTK, receptor tyrosine kinase; CSF-1R, colony-stimulating factor-1 receptor; SFK, Src-family kinase; JMD, juxtamembrane domain; IAA, iodoacetic acid.
Experimental Procedures

Reagents and Cell Lines—Anti-phosphotyrosine (pY100) antibody was from Cell Signaling. Anti-ubiquitin antibody (FK2) was from AFFINITY (U.K.). The anti-mouse CSF-1R peptide (965GDIQAQLQPNNYQ979) (CT) and the anti-pY559 CSF-1R peptide (555EGNSYTFIDTPQLYNEK572) antibodies were prepared as described (18). The goat anti-mouse CSF-1R C-terminal domain peptide (H) and the rabbit anti-mouse c-Cbl C-terminal peptide antibodies have been described previously (31). PP2 and SU6656 were purchased from Calbiochem. Human recombinant CSF-1 was a gift from Chiron Corporation, Emeryville, CA. Mouse MacCsf1r+/− (M−/−), M−/− WT and M−/− Y−/F macrophages, as well as M−/− YEF macrophages and their retrovirally transduced derivatives were maintained as described (18). Cloned MacCbl+/+ and MacCbl−/− cell lines were derived from bone marrow–derived macrophages (32) of Cbl−/− (33) and Cbl+/+ littermate control mice by immortalization with the SV-U19−5 retrovirus (34) as described (18). Clones were chosen that most closely resembled the primary macrophage populations from which they were derived. BAC1.2F5 macrophages have been previously described (35).

Site-directed Mutagenesis and Retroviral Constructs—The retroviral vector pMSCV-pgk-pac (36) and the ecotropic, replication-defective helper virus pSV-Ψ-MLV (37) cDNAs were gifts of Drs. R.G. Hawley (Holland Laboratory, American Red Cross, Derwood, MD) and O.N. Witte (University of California Los Angeles, Los Angeles, CA), respectively. A pGEM-2 plasmid containing an EcoRI fragment including the complete c-fms cDNA (nucleotides 1–36656, accession number NM_007779) was a gift from Dr. L.R. Rohrschneider (Fred Hutchinson Cancer Research Center, Seattle, WA). The human c-Cbl and 70Z-Cbl cDNAs were gifts from Dr. Wallace Y. Langdon of the Department of Pathology, University of Western Australia. Site-directed mutagenesis was performed using a kit according to the manufacturer’s instructions (Stratagene). All introduced mutations were confirmed by sequencing. The coding regions of mouse wild type (WT) or mutant CSF-1R cDNAs (K614A and cysteine mutations), human WT or mutant c-Cbl cDNAs were inserted into the MSCV-pgk-pac vector at the EcoRI site upstream of the pgk promoter driving the expression of puromycin resistance. The CSF-1R tyrosine add-back mutations were in pMSCV-IRES-GFP vector (38), a gift from Dr. A.W. Nienhuis (St Jude Children’s Research Hospital, Memphis, TN) and were used as described (18).

Retroviral Transfection of Macrophage Cell Lines—Human kidney 293T cells, cultured in 100-mm culture dishes with Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% FCS were co-transfected with the pSV-Ψ-E-MLV (12 μg) and either pMSCV-pgk-pac or pMSCV-IRES-GFP (12 μg) plasmids using calcium phosphate precipitation. The medium was changed to fresh 293T culture medium 24 h post-transfection. At 48-h post-transfection, the retroviral supernatant was harvested and filtered through a 0.45-μm filter. Subconfluent cultures of macrophages in 100-mm plates were incubated with the fresh retroviral supernatants in the presence of growth factors and 4 μg/ml polybrene for 24 h, prior to replacing the medium with fresh macrophage medium and culturing the cells for another 24 h. Depending on the plasmid, infected cells were selected either by adding 6 μg/ml of puromycin (Sigma) to the medium or by fluorescence-activated cell sorting as described (18). The pMSCV-pgk-pac-transduced cells were maintained in macrophage medium containing 2 μg/ml of puromycin. Total and cell surface CSF-1R levels were determined and cells with wild-type receptor levels isolated as previously described (18). For experiments, cells were cultured for no longer than 2 months.

CSF-1 Stimulation, Immunoprecipitation, and Western Blotting—Subconfluent (~70%) 100-mm dish cultures of macrophages were starved of growth factor for 20 h, then incubated with 360 ng/ml CSF-1 at 37 °C or 4 °C. Following incubation, the cells were rinsed in ice-cold PBS, lysed in Nonidet P-40 lysis buffer (1% Nonidet P-40, 10 mM Tris-HCl, 50 mM NaCl, 30 mM Na4P2O7, 5 mM NaF, 100 μM ZnCl2, 1 mM benzamidine, 10 μg/ml leupeptin, and 10 μg/ml aprotonin, pH 7.2) at 4 °C. For CSF-1R immunoprecipitation, the Nonidet P-40 lysates were incubated with 20 μl of immobilized anti-CSF-1R antibody CT (1–2 mg CT/ml packed Affi-gel 10 matrix beads (Bio-Rad)). For Cbl immunoprecipitation, the Nonidet P-40 lysates were mixed with 5 μg of anti-Cbl antibody and 20 μl of packed protein A-Sepharose 4B beads (Zymed Laboratories Inc.). Following incubation overnight at 4 °C, the immunoprecipitates were collected, washed and eluted with 10 μl of 3X SDS sample buffer at 65 °C for 10 min as described (18). Protein determinations, gradient (5–10% acrylamide) SDS-PAGE and Western blotting were performed as described previously (39). Blotted membranes were incubated with HRP Substrate (Millipore Corporation), the chemiluminescent signals recorded by ImageReader LAS-3000 (FUJIFILM, Tokyo, Japan) and analyzed with the software ImageGauge from FUJIFILM. Quantitation: phosphotyrosine and ubiquitination Western blot data from CSF-1R immunoprecipitation experiments were expressed as a proportion of WT levels, and corrected for receptor loading with reference to the CSF-1R Western blots. CSF-1R disulfide bonding was calculated from blots of unreduced gels as the ratio of the ~450-kDa band to the total level of mature receptor (excluding the ~135-kDa precursor).
Regulation of CSF-1R Activation

RESULTS

CSF-1 Binding Induces CSF-1R Covalent Modifications and Activation—The CSF-1R-deficient macrophage cell line, MacCsf1r−/− (M−/−) proliferates in GM-CSF. Retroviral expression of the wild-type (WT) CSF-1R in M−/− macrophages (M−/−, WT cells) reconstitutes CSF-1R-dependent survival, proliferation, differentiation, and morphological characteristics of primary bone marrow-derived macrophages (18). In response to CSF-1, M−/−, WT cells also exhibit CSF-1R tyrosine phosphorylation, ubiquitination, and degradation kinetics similar to those reported for primary macrophages and the BAC1.2F5 macrophage cell line. As shown in Fig. 1A (right panels), CSF-1R tyrosine phosphorylation was maximal by 1 min of stimulation, decreased at 3 min when receptor ubiquitination was maximal, and was barely detectable at 10 min when significant receptor degradation had occurred. CSF-1-induced CSF-1R interchain disulfide bonding was also examined by electrophoresis of the immunoprecipitates in the absence of β-mercaptoethanol (2ME) (Fig. 1A, left panels). As reported for BAC1.2F5 cells (40), a disulfide-bonded, tyrosine-phosphorylated dimeric receptor band of ~450 kDa, which was undetectable or in small amount when cells were not stimulated with CSF-1, was clearly visible by 1 min of stimulation, increased in intensity by 3 min and subsequently decreased as the receptor was degraded. The ~450-kDa band was not present when the samples were reduced prior to SDS-PAGE (Fig. 1A, right panels). In a previous study (40), we labeled macrophages with [35S]methionine and showed by V8 protease peptide mapping that the ~450-kDa form of CSF1-R observed on non-reducing gels is comprised solely of CSF-1R. Furthermore, an additional experiment indicates that the ~450-kDa species is comprised of approximately equal amounts of an ~165-kDa tyrosine-phosphorylated CSF-1R and an ~250-kDa ubiquitinated and tyrosine-phosphorylated CSF-1R (supplemental Fig. S1). Interestingly, the kinetics of CSF-1R disulfide bonding paralleled the kinetics of receptor ubiquitination (Fig. 1B).

The interchain disulfide bonding of CSF-1R may take place in cells immediately following receptor dimerization in the cell culture, or subsequently, during immunoprecipitation. To distinguish between these possibilities, CSF-1-stimulated BAC1.2F5 cells were lysed in SDS buffer containing iodoacetetic acid (IAA) to denature the protein, expose free sulfhydryls and to alkylate the free -SH groups to prevent disulfide bonding. The modified CSF-1Rs were immunoprecipitated and analyzed by Western blotting. If disulfide bonding of monomers within the CSF-1R dimer occurred in cells during stimulation with CSF-1, they would remain as disulfide-linked dimers following this treatment. Instead, as shown in Fig. 1C, while the ~450-kDa receptor band was still observed in the absence of IAA treatment, it disappeared when the free CSF-1R -SH groups were blocked by alklylation with IAA (compare lane 5 versus 2, 6 versus 3). This indicates that receptor disulfide bonding does not occur in the cells during stimulation, but subsequently, during immunoprecipitation. IAA had no effect on the formation of the ~450-kDa receptor band when samples were prepared in Nonidet P-40 lysis buffer (data not shown). While interchain disulfide bonding of the receptor does not occur in cells during stimulation, its occurrence during immunoprecipitation of CSF-1-stimulated lysates may be a reflection of conformational changes in the receptor associated with its activation.

To investigate whether CSF-1-induced receptor interchain disulfide bonding reflects CSF-1R activation, we expressed the kinase-dead mutant K614A CSF-1R and tyrosine phosphorylation-defective mutant YEF CSF-1R in M−/− cells. In the YEF mutant, all eight cytoplasmic tyrosines reported to be phosphorylated in the activated receptor were mutated to phenylalanines (18). When stimulated with CSF-1, both K614A and YEF CSF-1Rs failed to become activated, exhibiting neither tyrosine phosphorylation nor significant ubiquitination (Fig. 1D). Concomitantly, both of them failed to form the covalent dimers, indicating that the efficiency of CSF-1-induced CSF-1R interchain disulfide bonding can be used as a measure of receptor activation (Fig. 1E).

Tyr-559 Controls CSF-1-induced CSF-1R Covalent Modifications and Activation—We next examined the contributions of the individual cytoplasmic domain tyrosines to CSF-1-induced receptor tyrosine phosphorylation, ubiquitination, and activation. CSF-1R immunoprecipitates from stimulated M−/− cell lines individually expressing phenylalanine mutations of each of the eight tyrosines were analyzed by SDS-PAGE and Western blotting. As shown in Fig. 2A, Phe-544, Phe-559, Phe-697, Phe-721, and Phe-807 mutations significantly compromised CSF-1R tyrosine phosphorylation.
CSF-1R ubiquitination was slightly reduced in the Phe-697 and Phe-807 mutant receptors, but dramatically compromised in the Phe-559 mutant (Fig. 2B). Concomitantly, the Phe-559 mutation also abolished receptor disulfide bonding, while the Phe-697, Phe-721, and Phe-807 mutations had less effect (Fig. 2C), suggesting that among the eight tyrosines examined, Tyr-559 is most crucial for CSF-1-induced receptor covalent modifications and activation.

The sufficiency of Tyr-559 for CSF-1R ubiquitination and activation was investigated by creating four M−/− macrophage lines expressing mutant receptors, in which each of the four tyrosines affecting receptor covalent modifications (Tyr-559, Tyr-697, Tyr-721, and Tyr-807) were individually added back to the YEF backbone. M−/− macrophages expressing Y697AB, Y721AB, and Y807AB CSF-1Rs failed to undergo receptor tyrosine phosphorylation or exhibit significant receptor ubiquitination. In contrast, addition of Tyr-559 alone was sufficient to restore both these parameters with kinetics similar to those of M−/− WT cells (Fig. 3A). Because CSF-1R ubiquitination is important for receptor internalization and lysosomal degradation (25), we also examined the requirement and sufficiency of Tyr-559 for CSF-1-induced receptor degradation. Consistent with the finding that Tyr-559 was indispensable for CSF-1R ubiquitination, CSF-1-induced receptor degradation, evidenced by a reduction in the amount of the ~165-kDa band, was significantly reduced in M−/−.Y559F macrophages compared with M−/−.WT or M−/−.Y807F cells. In addition, the Y559AB CSF-1R displayed WT-like degradation kinetics whereas the Y807AB CSF-1R failed to undergo significant degradation (Fig. 3B). Similarly, the CSF-1-induced CSF-1R disulfide bonding was restored in the Y559AB receptor with kinetics indistinguishable from those of the WT CSF-1R, while the Y807AB CSF-1R failed to exhibit covalent dimers (Fig. 3C and D), suggesting that Y559AB alone rescued the appropriate CSF-1R conformational change that leads to receptor activation. The JMD Tyr-559, previously shown to be a critical switch regulating autoinhibition of receptor kinase activity (16–18), is hence both necessary and sufficient for CSF-1-induced CSF-1R ubiquitination, degradation, and activation. Consistent with the ability of the Y559AB to restore these parameters, Tyr-559 was phosphorylated in response to CSF-1 in M−/−.Y559AB cells (Fig. 3E). In support of the idea that Tyr-559 is the first tyrosine residue that is phosphorylated upon CSF-1 stimulation, we also observed that maximum phosphorylation of Tyr-559 preceded the attainment of maximum phosphorylation of Tyr-807, Tyr-697, and Tyr-721.
which was temporally correlated with the onset of CSF-1R ubiquitination (supplemental Fig. S2).

**Regulation of CSF-1R Activation**

**FIGURE 4. Requirement of c-Cbl for CSF-1-induced CSF-1R covalent modifications and activation.** Cells were stimulated and analyzed as described in the legend to Fig. 1A, A, CSF-1R tyrosine phosphorylation. B, assay of disulfide bonding of the tyrosine-phosphorylated CSF-1R (anti-phosphotyrosine immunoprecipitates). C, expression of c-Cbl in c-Cbl-transduced MacCbl−/− clones (clone 1, 5 × WT; clone 2, 3 × WT; clone 3, 1 × WT). D, CSF-1R ubiquitination in cells of clones 1 and 3. E and F, CSF-1R tyrosine phosphorylation and disulfide bonding of c-Cbl-transduced MacCbl−/− clones 1, 2, and 3.

**FIGURE 5. c-Cbl ubiquitin ligase activity is dispensable for CSF-1-induced CSF-1R covalent modifications and activation.** A, C, D, cells were stimulated and analyzed as described in the legend to Fig. 1A. A, CSF-1R tyrosine phosphorylation and disulfide bonding. B, overexpression of C381A-Cbl in MacCbl+/+ cells. C, CSF-1R tyrosine phosphorylation and ubiquitination. D, CSF-1R disulfide bonding.

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CSF-1-induced CSF-1R Covalent Modifications and Activation Are Dependent on c-Cbl Ligase Activity—CSF-1 stimulation increases the tyrosine phosphorylation of other cellular proteins in macrophages, one of which is c-Cbl, the major ubiquitin ligase for the CSF-1R (25, 31, 41). We have previously shown that CSF-1-induced tyrosine phosphorylation of both the CSF-1R and non-CSF-1R proteins was significantly reduced in c-Cbl−/- primary macrophages (25). To better understand the mechanism underlying this effect and the temporal relationship between receptor ubiquitination and activation, we established MacCbl−/− and MacCbl+/+ macrophage cell lines as described under “Experimental Procedures.” These cell lines possessed the proliferation and CSF-1 uptake characteristics previously reported for the primary macrophages (supplemental Fig. S3, A and B). Both MacCbl+/+ and MacCbl−/− macrophages expressed cell surface and total CSF-1R at similar levels (supplemental Fig. S3C), but consistent with our previous findings, MacCbl−/− macrophages displayed dramatically decreased CSF-1R tyrosine phosphorylation (Fig. 4A). Furthermore, when CSF-1R disulfide bonding was compared, the tyrosine-phosphorylated CSF-1R from MacCbl−/− cells failed to form the ~450-kDa covalent dimer, but instead remaining as the 165-kDa monomer (Fig. 4B), suggesting compromised CSF-1R activation in the context of c-Cbl deficiency.

To confirm that these phenotypes were caused by the loss of c-Cbl, we retrovirally transduced WT c-Cbl into the MacCbl−/− cells. Three independently arising clones were selected that expressed 1–5 times the endogenous level of c-Cbl (Fig. 4C). Cells expressing c-Cbl at WT or at 5 times WT levels both rescued CSF-1-stimulated CSF-1R ubiquitination (Fig. 4D) and the receptor disulfide bonding and tyrosine phosphorylation for all of the c-Cbl-reconstituted cell lines was fully restored (Fig. 4, E and F). These results indicate that c-Cbl is required for CSF-1R activation and tyrosine phosphorylation. Additional kinetic experiments at 4 °C demonstrated that c-Cbl-regulated CSF-1R ubiquitination is required for and coincident with the previously reported (31, 42) second wave of CSF-1R tyrosine phosphorylation (supplemental Fig. S4).

The effects of c-Cbl on CSF-1R tyrosine phosphorylation and activation could be catalytic, or via the action of c-Cbl as an adapter. To determine the requirement of c-Cbl catalytic activity, we expressed two c-Cbl RING domain mutants defective in mediating RTK ubiquitination, 70Z-Cbl, containing a 17-amino acid deletion, or C381A-Cbl, possessing a point mutation in the RING domain (43, 44), in MacCbl−/− macrophages. In contrast to WT-Cbl, 70Z-Cbl, and C381A-Cbl failed to rescue CSF-1R disulfide bonding or tyrosine phosphorylation (Fig. 5A). On the other hand, overexpression of C381A-Cbl in MacCbl+/+ cells (Fig. 5B) resulted in decreased CSF-1R tyrosine phosphorylation and ubiquitination (Fig. 5C), as well as decreased disulfide bond formation (Fig.
D), indicating a dominant-negative effect of C381A-Cbl.

These experiments prove that c-Cbl ligase activity is critical for CSF-1R activation and full receptor tyrosine phosphorylation.

**Inhibition of Src Family Kinase (SFK) Activity Inhibits CSF-1-induced CSF-1R Covalent Modifications and Activation**—Tyrosine phosphorylation of c-Cbl is associated with the activation of its ubiquitin ligase activity (21). As c-Cbl is tyrosine-phosphorylated in a SFK-dependent manner (30, 45), we examined the role of SFKs in CSF-1-induced receptor tyrosine phosphorylation and receptor interchain disulfide bonding and severely suppressed CSF-1R ubiquitination (Fig. 6B). Also, both SU6656 and PP2 significantly inhibited c-Cbl tyrosine phosphorylation (Fig. 6C). Consistent with the necessity of CSF-1R Tyr-559 for c-Cbl tyrosine phosphorylation and CSF-1R ubiquitination (Fig. 6C), the Y559F mutation in the CSF-1R abolished c-Cbl tyrosine phosphorylation (Fig. 6D) and consistent with the sufficiency of Tyr-559 for CSF-1R ubiquitination (Fig. 3A), c-Cbl tyrosine phosphorylation was significantly rescued in M/H11002/Y559F and M/H11002/Y559AB macrophages in response to CSF-1 stimulation.

**Interaction of the Major Kinase Domains of the CSF-1R Dimer Is Required for Receptor Activation and Full Receptor Tyrosine Phosphorylation, but Not for Receptor Ubiquitination**—Regardless of the fact that CSF-1R interchain disulfide bonding occurs during immunoprecipitation, we believe that the CSF-1-induced conformational change in the cytoplasmic domain of the CSF-1R juxtaposes free sulfhydryls of the ligand cross-linked receptor monomers so that they can engage in interchain disulfide bonding. As mapping the cysteines involved would indicate which intracellular domains of the receptors are brought into close proximity by this conformation change, we prepared a series of cysteine mutations of the CSF-1R as indicated in Fig. 7A and examined the effects of these mutations on CSF-1-induced receptor tyrosine phosphorylation, ubiquitination, and disulfide bonding. The selection of the amino acid replacement at each of these sites was based on (a) the commonly used substitutions for Cys, Ser, and Ala and (b) the appearance of the introduced amino acid at that site in other tyrosine kinases (46). Neither simultaneous mutation of the two cysteines in the extracellular domain (ECD) (A432A439) nor mutation of the three cysteines in the C terminus (CT) (S958S959L977) affected the efficiency of recep-

**FIGURE 6. Requirement of SFK for CSF-1-induced CSF-1R covalent modifications and activation.** A–C, CSF-1-starved M/H11002/WT cells were pretreated with 10 μM PP2 or 1 μM SU6656 for 1 h and then stimulated with CSF-1 for the indicated times at 37 °C. A and B, CSF-1R tyrosine phosphorylation, ubiquitination, and disulfide bonding. C, c-Cbl tyrosine phosphorylation. D, c-Cbl tyrosine phosphorylation in M/H11002/Y559F and M/H11002/Y559AB macrophages in response to CSF-1 stimulation.

**FIGURE 7. Effect of CSF-1-induced CSF-1R disulfide bonding on receptor tyrosine phosphorylation, ubiquitination, and degradation.** Cells were stimulated and analyzed as described in the legend to Fig. 1A. A, cysteine mutations examined. ECD, extracellular domain; TM, transmembrane domain; CT, C-terminal domain. B, effect of ATP-binding domain (SAA) mutations and major kinase domain (LSTV) mutations on CSF-1R disulfide bonding. C and D, effect of the major kinase domain mutant (LSTV) on CSF-1R tyrosine phosphorylation, ubiquitination (C), and degradation (D).
Regulation of CSF-1R Activation

A number of studies of class III RTKs have indicated that the JMD negatively regulates RTK activity. Mutations in the JMD of Flt3, c-Kit, and PDGFRα frequently result in constitutively activated RTKs that have been implicated in the pathogenesis of cancers (47, 48). Furthermore, a t(3;5)(p21;q33) translocation in acute megakaryoblastic leukemia has been shown to generate a transforming RNA-binding motif 6-CSF-1R fusion protein. Both the fusion protein and the region of the CSF-1R involved, lacking the JMD, have been shown to induce a myeloid proliferative disease with features of megakaryoblastic leukemia in a murine transplant model (49). More direct evidence in support of the JMD autoinhibitory model was that the JMD peptide of c-Kit inhibited the kinase activity of an active JMD-deficient c-Kit, whereas the tyrosine-phosphorylated JMD peptide was much less effective (50). In addition, the crystal structure of the unliganded Flt3 (51), c-Kit (52), and CSF-1 receptors (53) have provided details of the autoinhibited conformations of the kinase domains, documenting that the JMD inserts between the kinase N- and C-lobes and sterically locks the activation loop in its inactive conformation. Phosphorylation of Tyr-567 and Tyr-569 in the JMD of c-Kit disrupts these inhibitory interactions and allows the activation loop to adopt the active conformation (52). Mutation of these tyrosines is predicted to stabilize the autoinhibitory conformation, and indeed Phe-567/Phe-569 mutant of c-Kit significantly compromised receptor tyrosine phosphorylation and several downstream signaling pathways (54). Tyr-559 of the CSF-1R corresponds to Tyr-567 of c-Kit, and we (18) and other laboratories (16, 17) have found that the Phe-559 mutation significantly reduces CSF-1R kinase activity in vitro (18) and markedly suppresses CSF-1R tyrosine phosphorylation in vivo. Furthermore, Y559AB, but not the other tyrosine-add-back receptors (Fig. 3A), rescued receptor tyrosine phosphorylation, suggesting that phosphorylation of Tyr-559 occurs prior to phosphorylation of other tyrosine residues. While in the present study we observed less than complete rescue of Tyr-559 phosphorylation, this has since been explained by the finding that the Y544F mutation, part of the YEF background, compromises in vitro receptor kinase activity and autophosphorylation (18) and that when Tyr-544 is returned to the Y559AB background, in vitro kinase activity as well as wild-type levels of Tyr-559 phosphorylation, both in vitro and in vivo, are fully restored.3 In this context, it is relevant that Tyr-544 has only been reported to be phosphorylated in the v-fms oncoprotein (55).

The previous studies demonstrating that phospho-Tyr-559 recruits SFKs (19) and that Src associates with the ubiquitin ligase c-Cbl (30), support our results that Y559AB alone was sufficient for significant restoration of CSF-1-induced receptor ubiquitination and degradation. In addition, the reported associations of c-Cbl with Grb2 and PI3K (27), that respectively bind phospho-Tyr-697 and phospho-Tyr-721, are consistent with our observations that Phe-697, Phe-721 mutant receptors exhibited minor reductions in receptor ubiquitination. To our surprise, mutation of Tyr-974, the reported c-Cbl binding site on the CSF-1R (26), did not alter receptor ubiquitination. However, M−/−Y974F macrophages exhibited reduced spreading (18), suggesting that c-Cbl binding to Tyr-974 is involved in the regulation of cell spreading, but not receptor ubiquitination.

The ubiquitination of proteins can have diverse consequences. Apart from receptor degradation, we have previously demonstrated that formation of a ~250-kDa CSF-1R species in response to CSF-1 exhibited an increased specific activity of phosphate incorporation into tyrosine residues (42). In the absence of c-Cbl, the ~250-kDa receptor band failed to de-

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velop and the overall level of CSF-1R tyrosine phosphorylation was reduced (25) (Fig. 4A and supplemental Fig. S4). Because the conversion of the CSF-1R from ~165 kDa to ~250 kDa is due to c-Cbl-mediated ubiquitination (25), it is possible that CSF-1R ubiquitination itself contributes to the increased tyrosine phosphorylation of the ubiquitinated receptor. When the ubiquitin ligase activity of c-Cbl was either abolished directly by mutation in the RING domain (Fig. 5), or suppressed indirectly with SFK inhibitors (Fig. 6), CSF-1R tyrosine phosphorylation and ubiquitination were significantly reduced, consistent with the requirement of CSF-1R ubiquitination for the amplification of CSF-1R tyrosine phosphorylation. Recent studies of the NF-κB pathway have revealed a special role of Lys-63-linked polyubiquitination in facilitating TAK1 dimerization and activation, which phosphorylated a special role of Lys-63-linked polyubiquitination in

When the ubiquitin ligase activity of c-Cbl was either abolished directly by mutation in the RING domain (Fig. 5), or suppressed indirectly with SFK inhibitors (Fig. 6), CSF-1R tyrosine phosphorylation and ubiquitination were significantly reduced, consistent with the requirement of CSF-1R ubiquitination for the amplification of CSF-1R tyrosine phosphorylation. Recent studies of the NF-κB pathway have revealed a special role of Lys-63-linked polyubiquitination in facilitating TAK1 dimerization and activation, which phosphorylated a special role of Lys-63-linked polyubiquitination in

Recent studies of the NF-κB pathway have revealed a special role of Lys-63-linked polyubiquitination in facilitating TAK1 dimerization and activation, which phosphorylates and activates IκB kinases (56). Similarly, c-Cbl-catalyzed ubiquitination favors CSF-1R dimerization that augments receptor activation and tyrosine phosphorylation. However, c-Cbl may catalyze the ubiquitination of proteins other than the CSF-1R that are also involved. For example, it has been reported that c-Cbl itself is also ubiquitinated in response to CSF-1, probably autocatalytically (41). Moreover, as a multi-domain adaptor protein, it is also possible that c-Cbl transports a kinase or another signaling molecule to the CSF-1R that is responsible for the increase of receptor tyrosine phosphorylation. It has also been reported that c-Cbl can directly bind to the phospho-Tyr569 site of c-Kit (equivalent to Tyr-559 of CSF-1R) in vitro (57), so that it is possible that c-Cbl recruits SFKs to the CSF-1R and that they amplify CSF-1R tyrosine phosphorylation. Thus whether the increased receptor tyrosine phosphorylation can be ascribed directly to the ubiquitination of the CSF-1R, or other proteins, remains to be determined.

Ligand-induced interchain disulfide bonding of receptor subunits has been reported for several receptors, including the growth hormone receptor (58), the interleukin-3 receptor (59), the PDGFRβ (60) and the CSF-1R (40). In the present study, we demonstrated that CSF-1R interchain disulfide bonding occurs following cell lysis. However, because it was dependent on CSF-1 binding and required both CSF-1R intrinsic kinase activity and the initial phosphorylation of Tyr-559 to relieve the autoinhibition, we used it as a measure of the activated conformation of the CSF-1R. This enabled us to use cysteine mutagenesis to identify the domains that were brought together to amplify tyrosine phosphorylation in the activated receptor dimer. The mutagenesis experiments have shown that cysteines in the kinase domain, in both the ATP-binding domain and the major kinase domain, are indispensable for CSF-1R disulfide bonding. Mutation of the three cysteines in the ATP-binding domain, SAA, completely abolished not only receptor disulfide bonding, but also receptor tyrosine phosphorylation (supplemental Fig. S6). Therefore, as expected, M−/−SAA macrophages failed to survive or proliferate in response to CSF-1 (data not shown). Furthermore, it has been shown in activated c-Kit that after ATP-binding, Cys-672 of the ATP-binding domain (equivalent to Cys-664 of the CSF-1R) forms hydrogen bonds with the adenine N1 atom of ADP (61). It is therefore likely that the SAA mutation distorted the structure of the ATP-binding domain, affecting the ATP binding and/or kinase activation required for receptor tyrosine phosphorylation and consequently for the conformational change in the major kinase domain.

Mutation of the cysteines in the major kinase domain also substantially suppressed disulfide bonding and receptor tyrosine phosphorylation. It is possible that the LSTV mutations in the major kinase domain cause the conformational alterations that compromise the kinase activity, other than those associated with the disruption of domain interaction and that these negatively impact receptor tyrosine phosphorylation. We consider this unlikely for the following reasons. In the first place, the LSTV mutant receptor was efficiently tyrosine phosphorylated in vitro (supplemental Fig. S7). Secondly, in contrast to M−/−SAA macrophages, there was no significant effect of the LSTV mutations on CSF-1-regulated cell proliferation (data not shown). Thirdly, when the interchain disulfide bonding of the wild type CSF-1Rs in MacCbl−/−cells or SFK inhibitor-treated cells was suppressed, CSF-1R tyrosine phosphorylation was also decreased. Lastly, any cysteine add-back to the LSTV backbone completely rescued CSF-1R disulfide bonding (supplemental Fig. S8). These results suggest that the interaction of two major kinase domains within the CSF-1R dimer provide a conformational advantage for receptor activation and full tyrosine phosphorylation.

The fact that M−/−LSTV macrophages are not significantly compromised in their proliferative response to CSF-1 implies that Tyr-559/SFK/c-Cbl-mediated amplification of CSF-1R tyrosine phosphorylation is not important for its competence to stimulate the WT proliferative response. Consistent with this, in recent experiments we found that addition of Tyr-544, Tyr-559, and Tyr-807 back to the YEF backbone is sufficient to fully restore the WT proliferative response under conditions in which Tyr-807 is phosphorylated at a fraction of the level observed in the WT CSF-1R, but that phosphorylation of additional tyrosines mediate morphological responses (18) and chemotaxis to CSF-1.

The present studies demonstrate that third party molecules, other than tyrosine kinases and protein tyrosine phosphatases, play important roles in RTK phosphorylation and full receptor activation. Variation in the expression of the third party molecules that regulate receptor function in this fashion can therefore be expected to contribute significantly to the variations in cell context that have been noted for RTKs in general and for the CSF-1R in particular (14). The identification of these molecules and the covalent CSF-1R modifications that mediate open new areas of investigation. Clearly the nature, symmetry and the role of ubiquitination of the CSF-1R at particular sites need to be addressed. Most importantly, the structural consequences of these receptor modifications require visualization to properly understand the mechanisms by which they regulate activation.

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