The Src-like Adaptor Protein 2 Regulates Colony-stimulating Factor-1 Receptor Signaling and Down-regulation*

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 Src-like adaptor protein 2 (SLAP-2) is a hematopoietic adaptor protein previously implicated as a negative regulator of T-cell antigen receptor (TCR)-mediated signaling. SLAP-2 contains an SH3 and an SH2 domain, followed by a unique carboxyl-terminal tail, which is important for c-Cbl binding. Here we describe a novel role for SLAP-2 in regulation of the colony-stimulating factor 1 receptor (CSF-1R), a receptor tyrosine kinase important for growth and differentiation of myeloid cells. SLAP-2 co-immunoprecipitates with c-Cbl and CSF-1R in primary bone marrow-derived macrophages. Using murine myeloid cells expressing CSF-1R (FD-Fms cells), we show that SLAP-2 is tyrosine-phosphorylated upon stimulation with CSF-1 and associates constitutively with both c-Cbl and CSF-1R. In addition, we show that expression of a dominant negative form of SLAP-2 impairs c-Cbl association with the CSF-1R and receptor ubiquitination. Impaired c-Cbl recruitment also correlated with changes in the kinetics of CSF-1R down-regulation and trafficking. CSF-1-mediated differentiation of FD-Fms cells and activation of downstream signaling events was also enhanced in cells stably expressing dominant negative SLAP-2. Together, these results demonstrate that SLAP-2 plays a role in c-Cbl-dependent down-regulation of CSF-1R signaling.

The colony-stimulating factor 1 receptor (CSF-1R) is a member of the type III receptor tyrosine kinase (RTK) family, which includes c-Kit, platelet-derived growth factor receptor α and β, and Flt3. All of the functions of colony-stimulating factor-1 (CSF-1) are mediated by CSF-1R, which is the primary regulator of the common myeloid lineage consisting of osteoclasts and mononuclear phagocytes (monocytes and macrophages) in vivo.

Ligand binding induces CSF-1R dimerization and autophosphorylation on tyrosine residues that regulate both kinase activity and mediate binding to downstream SH2 and PTB domain-containing proteins. Recruitment of such proteins leads to activation of signaling cascades involved in determining the biological outcomes of CSF-1R activation as well as receptor down-regulation (6–8).

A number of adaptor proteins, including Gads/Mona, FMIP, DOK-2, and c-Cbl, have been implicated as negative regulators of CSF-1R signaling. c-Cbl possesses a variant SH2 domain (TKB domain) that mediates binding to activated tyrosine kinases and a RING finger domain demonstrated to confer E3 ubiquitin ligase activity, which promotes the ubiquitination of activated tyrosine kinases (9, 10). In c-Cbl-deficient macrophages, both CSF-1R ubiquitination and endocytosis of the ubiquitinated receptor are impaired (11). Subsequent to CSF-1R ubiquitination, activated CSF-1R is degraded intracellularly (12). The degradation of ligand-stimulated CSF-1R normally occurs rapidly, and the half-life of the CSF-1R decreases from 3 h to 5 min upon ligand binding (13, 14). Mutation of the tyrosine residue within the c-Cbl binding site (Tyr-969 in human CSF-1R) has been frequently observed in myelodysplasia and acute myeloblastic leukemia (15). Similarly, the transforming viral homologue of CSF-1R, v-Fms, lacks the c-Cbl binding site. Importantly, re-addition of the c-Cbl binding site to v-Fms decreases its transforming activity (16).

Src-like adaptor protein family proteins, SLAP and SLAP-2, have an amino-terminal myristoylation signal required for association with cell membranes, closely juxtaposed SH3 and SH2 domains, and a unique carboxyl-terminal region that mediates association with c-Cbl. Both SLAP and SLAP-2 have been demonstrated to function as negative regulators of T-cell antigen receptor (TCR) signaling via a mechanism requiring the c-Cbl binding region (17–19). In addition to c-Cbl, SLAP-2 associates with the Zap-70 and Syk cytoplasmic tyrosine kinases and promotes their degradation when co-expressed. Recent work has shown that SLAP functions as a negative regulator of the TCR by promoting c-Cbl-dependent ubiquitination of TCR chain ζ and down-regulation of the CD3 complex (17, 20, 21). In addition, both SLAP and SLAP-2 have been implicated as negative regulators of B-cell receptor signaling (19, 22), and avian SLAP has been demonstrated to interfere with erythropoietin signaling in erythroblasts (23).

Although SLAP and SLAP-2 have been implicated in the regulation of antigen receptor signaling, SLAP was originally dis-
SLAP-2-dependent Regulation of CSF-1R

covered in the context of RTK signaling (24) and ectopic expression of SLAP in fibroblasts shown to inhibit platelet-derived growth factor receptor-induced proliferation, suggesting that SLAP may also act as a negative regulator of RTK signaling (25, 26). Both SLAP and SLAP-2 are expressed in lymphoid tissues and cell lines, whereas SLAP-2 is also abundantly expressed in myeloid cell lines such as KG1a, OCI, AML-2, -3, and -5 (27). A recent study by Manes et al. (28) demonstrated that SLAP-2 is expressed in murine bone marrow-derived macrophages and suggested a potential role for SLAP-2 in CSF-1 signaling. Here we provide evidence that SLAP-2 plays a role in c-Cbl recruitment to activated CSF-1 receptors and consequent down-regulation of CSF-1R signaling by promoting internalization and degradation of activated receptors.

EXPERIMENTAL PROCEDURES

Bacterial and Mammalian Expression Constructs—Wild-type SLAP-2 and SLAP-2 mutants have been previously described (17). SLAP-2 G2A contains a point mutation changing amino acid 2 to alanine, SH2* has a point mutation changing the arginine residue at amino acid position 120 to lysine, and in the ΔC truncation mutant a stop codon was introduced such that it lacks the last 70 carboxyl-terminal amino acids. SLAP-2 GST fusion proteins were prepared as previously described (17). SLAP and SLAP-2 (WT, G2A, and SH2*) constructs were subcloned into the HSC retroviral vector (provided by Dr. James Ellis) at SalI and BamHI sites for use in preparation of ecotropic virus.

FD-Fms Cell Culture, Cell Stimulation, and Lysis—Murine myeloid FDC-P1 cells expressing wild-type Fms (FD-Fms cell line) were kindly provided by Dr. Larry R. Rohrschneider (Fred Hutchinson Cancer Research Center). FD-Fms cells were cultured at 37 °C, 5% CO2 in DMEM supplemented with 10% fetal bovine serum (FBS) and 5% WEHI cell supernatant (as a source of IL-3). WEHI supernatant was prepared by culturing cells in RPMI 1640 medium and 10% FBS for 7–10 days. Before FD-Fms cells were stimulated with CSF-1, they were starved of IL-3 by washing twice with phosphate-buffered saline (PBS) and culturing in DMEM with 10% FBS for 5–6 h. Cells were then suspended at 10–20 × 106 cells/ml in serum-free DMEM and 100 ng/ml rmCSF-1 (R&D Systems) at 37 °C for various times before pelleting and lysis in cold Nonidet P-40 lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Nonidet P-40, 1 mM EGTA (pH 8.0)) freshly supplemented with complete protease inhibitors (Roche Applied Science), 1 mM sodium orthovanadate, and 10 mM NaF.

Retroviral Infection of FD-Fms Cells—The ecotropic Phoenix packaging cell line (generously provided by Dr. Ian Clarke) was cultured in DMEM with 10% FBS and transiently transfected using Lipofectamine (Invitrogen) with the HSC IRES-eGFP retroviral vectors containing wild-type (WT), and mutant forms SLAP-2. Viral particle containing supernatants were collected, and carryover cells/debris was removed by passing through a 45-μm filter (Millipore) and used directly for spin infection of FD-Fms cells. Briefly, 1.5 × 10^6 FD-Fms cells were suspended in 3 ml of retroviral supernatant supplemented with 8 μg/ml Polybrene (Hexadimethrine Bromide, from Sigma). Cells were centrifuged in 50-ml Falcon tubes at 1800 rpm (650 × g) for 90 min at room temperature. Cells were resuspended, and a second spin was performed. The cells were suspended in fresh viral supernatant plus Polybrene and incubated overnight at 32 °C and 5% CO2. Media was then changed back to DMEM with 10% FBS and 5% WEHI supernatant, and cells were expanded for 4 days. GFP-positive cells were collected using fluorescence-assisted cell sorting, expanded, and sorted as necessary to select comparable levels of WT, G2A, and SH2* SLAP-2 protein levels.

Differentiation of FD-Fms Cells—FD-Fms cell lines (HSC, G2A, WT, and SH2*) were washed twice, suspended in DMEM with 10% FBS and seeded at 5000 cells/ml in DMEM with 10% FBS and 2500 units of hCSF-1/ml (provided by Dr. E. Richard Stanley) in 10-ml cultures. Cells were analyzed by flow cytometry using FlowJo software (Tree Star) on day 3 of culture to measure side-scatter (SSC) and CD11b (Mac-1). Cells were incubated with Fc block (anti CD16/32 antibody) prior to incubation with PE-anti-CD11b or isotype control (Rat IgGb2b) antibodies. Dead cells were excluded from the analysis by staining with propidium iodide.

Preparation and Stimulation of Primary Bone Marrow-derived Macrophages—Primary bone marrow-derived macrophage were prepared from the femur bone marrow of 6-week-old mice. To remove differentiated cells, bone marrow was suspended in RPMI supplemented with 10% fetal bovine serum (Invitrogen), 5 units of penicillin C/ml, and 5 mg of streptomycin sulfate/ml for 2 h. Adherent cells were discarded, while non-adherent cells were counted, and plated at 1.25 × 10^6 cells/ml in DMEM with 10% FBS and 2,500 units of recombinant hCSF-1/ml for 7–10 days in 10-cm culture plates. Adherent bone marrow-derived macrophages were starved of CSF-1 for 24 h and stimulated by washing once with serum-free DMEM at 37 °C, and then stimulated for various times in serum-free DMEM supplemented with 100 ng/ml rmCSF-1. Stimulated cells were subsequently lysed in Nonidet P-40 buffer, and soluble protein lysates were used for immunoprecipitations as described.

Immunoprecipitation and Western Blotting—Cells were lysed in Nonidet P-40 lysis buffer with complete protease inhibitors and 1 mM sodium orthovanadate. Lysates were cleared by centrifugation at 14,000 rpm (20,800 × g) at 4 °C for 10 min and precleared by incubation with 50 μl of 20% (v/v) protein G-Sepharose beads or with protein A-Sepharose beads (Sigma) at 4 °C for 30 min. Precleared lysates were incubated with antibodies (described below) and either protein G- or protein A-Sepharose beads as described above and then incubated at 4 °C with gentle rotation for 90 min. Immune complexes were washed four times in 1 ml of cold Nonidet P-40 wash buffer (Nonidet P-40 lysis buffer with 0.1% Nonidet P-40, without EGTA, and 20 mM HEPES (pH 7.5)), and bound proteins were eluted by boiling for 5 min in 2× SDS sample buffer. Eluted proteins were resolved by SDS-PAGE on 10% gels. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences) and incubated in blocking solution for a minimum of 30 min prior to the addition of antibodies. Bound antibodies were detected by using enhanced chemiluminescence reagent (ECL, PerkinElmer Life Sciences).
In Vitro Binding Assays and Far Western—GST fusion proteins were expressed in DH5α bacteria and purified on glutathione-Sepharose beads (Amersham Biosciences). In vitro binding assays were done with FD-Fms cell lysates (1 mg/ml) either unstimulated or stimulated with rmCSF-1. Lysates were incubated with 4 μg of GST fusion proteins for 90 min at 4 °C. Following several washes, bound proteins were eluted in 2× SDS sample buffer and resolved by SDS-PAGE. Membranes were blocked with Coomassie Blue dye to ensure equal loading of fusion proteins. For the far Western, GST-SLAP-2 was eluted from Sepharose beads by incubating beads in 3 mg/ml glutathione in 50 mM Tris, pH 8.0, then dialyzing in PBS before quantifying. Anti-CSF-1R immunoprecipitates from the FD-Fms cell line were subjected to SDS-PAGE, and transferred to Nitrocellulose (Amersham Biosciences) membranes. The membranes were blocked in TBST (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Tween 20) with 5% (w/v) skim milk powder for 1 h at room temperature, followed by incubation with 10 μg/ml GST-SLAP-2 in blocking solution supplemented with 10 mM dithiothreitol overnight at 4 °C. Following three 5-min washes with TBST, membranes were incubated with anti-GST antibody in blocking solution for 1 h, washed again, then incubated with protein-A linked horseradish peroxidase for 40 min. Finally, membranes were washed (4 × 5 min and 1 × 10 min) with TBST before ECL detection was used.

Antibodies—Polyclonal SLAP-2 was produced as previously described (17). 10 μl (3 ug) of affinity-purified SLAP-2 antibody was used in immunoprecipitation experiments, and a concentration of 0.3 μg/ml was used for immunoblotting. The following antibodies and conditions were used: anti-phosphotyrosine 4G10 (UBI), 1:1000 for Western blotting; rabbit anti-STAT3 (UBI), 1:1000; anti-phospho-STAT3 (UBI), 1:1000; a 1:1 mix of mouse anti-c-Cbl (clone 17, BD Transduction Laboratories) and clone 7G10 (UBI), 1:1000; rabbit anti-Fms (CSF-1R) (UBI) was used at 2 μl for immunoprecipitation; 1:1 mix of rabbit anti-pY-CSF receptor (CSF-1R) phosphotyrosine 723 and phosphotyrosine 809 (Cell Signaling), 1:1000; rabbit anti-CSF-1R clone C-20 (Santa Cruz Biotechnology), 1:1000; anti-ubiquitin (Covance) 1:1000; anti-transferrin receptor (Zymed Laboratories Inc.) 1:1000; anti-GST (UBI), 1:1000; sheep anti-mouse antibody (1:6000 dilution); and protein A (1:3000 dilution) conjugated to horseradish peroxidase were used to detect bound primary mouse monoclonal antibodies and polyclonal antibodies, respectively. Blocking solutions varied according to the manufacturer’s recommendations. PE-conjugated anti-CD11b (clone M1/70), rat IgG2b, and anti-CD32/16 antibodies used for flow cytometry were purchased from eBioscience.

Subcellular Fractionation—FD-Fms cells (2.5 × 10^7) were washed with phosphate-buffered saline (PBS) and lysed in 1 ml of hypotonic lysis buffer (10 mM Tris-HCl (pH 8.0), 1 mM MgCl₂) containing complete protease inhibitors and 1 mM sodium orthovanadate. Cells were passed through a Dounce homogenizer until all cells were lysed, as checked by trypan blue staining. Lysed cells were adjusted back to isotonic conditions by the addition of 5 mM NaCl to a final concentration of 150 mM. Lysates were centrifuged at 3,000 rpm (900 × g) for 10 min at 4 °C. The pellet representing the nuclear fraction was discarded. The supernatant from the first spin was centrifuged in a Beckman tabletop ultracentrifuge by using the TLA-45 rotor at 43,000 rpm (100,000 × g) for 30 min at 4 °C. The pellet representing the membrane fraction was resuspended in extraction buffer (1% SDS, 1% Triton X-100, 1% sodium deoxycholate) in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM MgCl₂. The supernatant was adjusted to 0.1% SDS-0.1% Triton X-100/0.1% sodium deoxycholate.

Biotinylation of Surface Proteins—FD-Fms cells were stimulated for various times with 100 ng/ml rmCSF-1 (R&D Systems) at 37 °C. After stimulation, cells were cooled on ice for 5 min and washed twice with cold PBS. Cells were then incubated with biotin (EZ-Link NHS-SS-Biotin, Pierce) diluted at 0.2 mg/ml in biotinylation buffer (154 mM NaCl, 10 mM HEPES, 3 mM KCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 10 mM glucose, pH 7.6) for 1 h at 4 °C. After labeling, cells were washed twice with cold PBS, blocked for 5 min in DMEM (10% FBS, 100 mM glucose) at 4 °C, washed twice in cold PBS, and lysed in PLC lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton-X-100, 1 mM EGTA (pH 8.0) freshly supplemented with complete protease inhibitors (Roche Applied Science), 1 mM sodium orthovanadate, and 10 mM NaF). Biotin-labeled proteins were recovered by incubating ~1 mg of lysate with 30 μl of streptavidin beads (Immunopure Immobilized Streptavidin, Pierce) overnight. Beads were washed 4× with PLC wash buffer and boiled in SDS sample buffer. Samples were analyzed by Western blotting against CSF-1R.

RESULTS

SLAP-2 Associates with c-Cbl and CSF-1R in Primary Bone Marrow Macrophages and in FD-Fms Cells—To investigate the role of SLAP-2 in CSF-1R signaling, we performed immunoprecipitations from untreated or CSF-1-stimulated bone marrow-derived macrophage cell lysate using anti-SLAP-2 and anti-CSF-1R antibodies. Anti-phosphotyrosine Western blotting revealed that several tyrosine-phosphorylated proteins co-precipitated with SLAP-2 following CSF-1 stimulation compared with pre-immune control serum (Fig. 1A, upper panel). The tyrosine-phosphorylated protein of ~120 kDa was identified as c-Cbl (Fig. 1A, lower panel). Notably the association of SLAP-2 with c-Cbl was observed in unstimulated cells but was enhanced following treatment with CSF-1. A similar constitutive association between SLAP-2 and c-Cbl has also been observed in primary thymocytes (17). A tyrosine-phosphorylated protein of 180 kDa that co-migrated with the CSF-1R was also observed (Fig. 1A, upper panel), although anti-CSF-1R antibody was not sensitive enough to confirm its identity. A phospho-specific antibody (pY-CSF-1R) directed against phosphorylated tyrosine residues Tyr-807 and Tyr-721 on the activated protein of 180 kDa was identified as CSF-1R (Fig. 1B). Due to a high level of background, and the low level expression, endogenous SLAP-2 could not be detected upon subsequent re-blotting of the SLAP-2 immunoprecipitates (data not shown).

To further investigate the role of SLAP-2 in CSF-1R signaling, we utilized the FDC-P1 myeloid cell line, FD-Fms, which expresses wild-type murine CSF-1R (29, 30). Immunoprecipitation of endogenous SLAP-2 from lysates of unstimulated or CSF-1-stimulated cells probed with anti-SLAP-2 revealed...
bands corresponding to the p28 and p25 forms of SLAP-2 that have previously been described in hematopoietic cell lines and tissues (Fig. 1C, bottom panel) (17). Endogenous SLAP-2 was specifically co-immunoprecipitated with tyrosine-phosphorylated proteins of 120 and 180 kDa following CSF-1 stimulation (Fig. 1C, top panel). Re-blotting identified the 120-kDa band as c-Cbl and showed that C-Cbl associated with SLAP-2 prior to CSF-1R stimulation (Fig. 1C, middle panel). Again, anti-CSF-1R antibody was not sensitive enough to detect the co-immunoprecipitated CSF-1R; however, using the pY-CSF-1R antibody we were able to demonstrate that the CSF-1R is co-precipitated with SLAP-2 in FD-Fms cells (Fig. 1D).

To determine which regions of SLAP-2 mediate its association with c-Cbl and CSF-1R, GST-pulldown assays were carried out using lysates from FD-Fms cells. Recombinant wild-type (WT) GST-SLAP-2 associated with tyrosine-phosphorylated proteins of similar size observed in SLAP-2 immunoprecipitates, and subsequent immunoblotting confirmed the identity of c-Cbl and CSF-1R, respectively (Fig. 2B). Similarly, a GST fusion of the related SLAP molecule also bound to c-Cbl and the CSF-1R. In this system, SLAP-2 association with the receptor appeared to be constitutive, whereas SLAP binding to CSF-1R was strongly induced by CSF-1 stimulation (Fig. 2B). In addition, c-Cbl binding to
both GST-SLAP-2 and GST-SLAP was strongly induced by CSF-1 stimulation (Fig. 2B).

A GST-SLAP-2-ΔC fusion protein, which lacks the entire carboxyl-terminal region (ΔC, Fig. 2A), was unable to associate with c-Cbl, consistent with previous studies that indicated the C-terminal region of SLAP-2 mediates its association with c-Cbl (Fig. 2B, second panel) (17, 19, 27). This form of SLAP-2 also showed a significant reduction in CSF-1R binding suggesting that this region also stabilizes SLAP-2 binding to the receptor. A mutant form of SLAP-2 with an inactivating mutation in the SH2 domain (SH2*, Fig. 2A) failed to bind CSF-1R (Fig. 2B, third panel), suggesting that the SLAP-2 SH2 domain is required for receptor association in the presence or absence of growth factor stimulation. These observations contradict those of Manes et al., who observed that neither mutation of the SH2 domain nor deletion of the carboxyl terminus had an effect on CSF-1R binding when co-expressed in HEK293T cells (28). To address whether the association between SLAP-2 and CSF-1R is direct, CSF-1R was immunoprecipitated from FD-Fms cells before (0') and 2 min after (1') CSF-1 stimulation. As a control, similar conditions were used for a nonspecific rabbit IgG. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, incubated with recombinant GST-SLAP-2, and subsequently immunoblotted with anti-GST antibodies. The same membrane was stripped and re-immunoblotted with anti-CSF-1R antibody as indicated.

**FIGURE 2.** SLAP-2 associates in vitro with c-Cbl and CSF-1R in FD-Fms cells. A, schematic of SLAP-2 GST fusion proteins used. Wild-type SLAP-2 (WT), SLAP-2 with an inactivating mutation in the SH2 domain (SH2*), and a truncated form of SLAP-2 (ΔC) were used in the GST pulldown experiment. B, SLAP and SLAP-2 associate with similar subsets of tyrosine-phosphorylated proteins in vitro. Purified, immobilized GST fusion proteins were incubated with FD-Fms cell lysates before (0') and after (1') CSF-1 stimulation. Immunoblotting was performed with the indicated antibodies. Equivalent concentrations of GST fusion proteins were evaluated by Coomassie Blue staining (bottom panel). L, soluble cell lysates. C, SLAP-2 associates directly with CSF-1R. A modified Far Western was carried out, in which CSF-1R was immunoprecipitated from FD-Fms cells before (0') and 2 min after (1') CSF-1 stimulation. As a control, similar conditions were used for a nonspecific rabbit IgG. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, incubated with recombinant GST-SLAP-2, and subsequently immunoblotted with anti-GST antibodies. The same membrane was stripped and re-immunoblotted with anti-CSF-1R antibody as indicated.
SLAP-2-dependent Regulation of CSF-1R

SLAP-2 Is Tyrosine-phosphorylated upon CSF-1 Stimulation—When endogenous SLAP-2 was immunoprecipitated from lysates of CSF-1-stimulated FD-Fms cells we observed slower migrating species in addition to the two SLAP isoforms expressed in these cells. (Fig. 3A). Previously we and others (28) reported that SLAP-2 is phosphorylated on serine residues when ectopically expressed, but tyrosine phosphorylation had not been reported. To test whether the slower migrating SLAP-2 species observed following CSF-1 stimulation is also tyrosine-phosphorylated, SLAP-2 was immunoprecipitated from a cell line engineered to overexpress WT SLAP-2 and immunoblotted with anti-phosphotyrosine (Fig. 3B). A tyrosine-phosphorylated band that co-migrated with the transient higher molecular weight form of SLAP-2 was observed, suggesting that SLAP-2 is transiently tyrosine-phosphorylated upon activation of the CSF-1R. When SLAP-2 immunoprecipitates were incubated with phosphatase, the anti-phosphotyrosine reactivity was lost and accompanied by a change in electrophoretic mobility (Fig. 3C). Taken together, these results suggest that SLAP-2 becomes tyrosine-phosphorylated upon CSF-1 stimulation.

SLAP-2 Modulates c-Cbl Association and Down-regulation of the CSF-1R—To examine the role of SLAP-2 in CSF-1R signaling and down-regulation, we created FD-Fms stable cell lines expressing wild-type SLAP-2 (WT) or forms of SLAP-2 harboring mutations that disrupt myristoylation (G2A) and inactivate the SH2 domain (SH2*). Stably transduced cell lines (HSC, WT, G2A, and SH2*) were stimulated with CSF-1 at 4 °C (to slow the kinetics of receptor down-regulation), and membrane fractions were isolated and blotted for CSF-1R and activated CSF-1R (pY-CSF-1R). In cells overexpressing WT SLAP-2, a decrease in the total receptor levels and the amount of phosphorylated CSF-1R in the membrane fraction was observed at all time points following CSF-1 stimulation compared with vector alone (HSC) (Fig. 4A, left panel). In contrast, in cells expressing SLAP-2 SH2*, a prolonged presence of the activated receptor in the membrane fraction as compared with cells expressing either the HSC vector or G2A SLAP-2 was observed (Fig. 4A, right panel). These results imply that SLAP-2 overexpression can promote receptor down-regulation, whereas introduction of a dominant interfering form of SLAP-2 (SH2*) interferes with down-regulation of the activated receptor.

The E3 ligase c-Cbl rapidly associates with the CSF-1 receptor upon stimulation and is a critical event leading to receptor ubiquitination and down-regulation. To examine the effect of SLAP-2 on the association between c-Cbl and CSF-1R, the receptor was immunoprecipitated from HSC and SLAP-2 WT-, SH2*-, and G2A-expressing cell lines before and after stimulation with CSF-1. Compared with controls (HSC), immunoblotting revealed that overexpression of WT SLAP-2 or the G2A mutant had no effect on the association of CSF-1R and c-Cbl after 1 min of CSF-1 (Fig. 4B). However, in cells expressing SLAP-2 SH2* a dramatic decrease in receptor-associated c-Cbl was observed (Fig. 4B, right panel), further supporting the notion that SLAP-2 plays a role in c-Cbl mediated down-regulation of the CSF-1R.

c-Cbl has been demonstrated to be important for ubiquitination of the CSF-1R (11). Because the SH2 mutant of SLAP-2 (SH2*) still binds c-Cbl but is uncoupled from the CSF-1 receptor, we examined whether its expression affects CSF-1R ubiquitination. FD-Fms cell lines (HSC, WT, G2A, and SH2*) were stimulated with CSF-1 at 37 °C, and CSF-1R was immunoprecipitated and blotted with an anti-ubiquitin antibody. CSF-1R ubiquitination was observed by 3 min, and ubiquitinated species were barely detectable by 30 min (Fig. 5A). Overexpression of WT SLAP-2 appeared to modestly increase ubiquitination of CSF-1R at 3 min but did not affect subsequent down-regulation and degradation. In cells expressing SLAP-2 SH2* the level of CSF-1R ubiquitination was dramatically reduced at the 3-min time point in comparison to control (HSC) and SLAP-2 G2A-expressing cells (Fig. 5A).
To examine if the observed differences in ubiquitination of CSF-1R correlated with changes in receptor internalization and degradation we performed surface biotinylation experiments. FD-Fms cell lines were stimulated with CSF-1 as indicated and then labeled with biotin (Fig. 5B). Biotinylated surface proteins were then isolated, and the presence of CSF-1R was determined by Western blotting. All the cell lines showed a loss of surface CSF-1R after stimulation, consistent with the internalization and degradation of activated receptors. However, in cells overexpressing SLAP-2 SH2* there was a significant retention of CSF-1R at the cell surface at 1 min, suggesting that activation-induced internalization of the receptor was impaired (Fig. 5B).

In addition, after a reduction at 5 min, an increase in the cell surface levels of CSF-1R was observed after 10 min in the SLAP-2 SH2*-expressing cells, whereas in the control and WT- or G2A-expressing cells lines biotinylated cell surface receptor was almost completely absent (Fig. 5B). This observation is consistent with a model in which expression of SLAP-2 SH2* interferes with routing of the receptor to the lysosome and promotes the recycling of the CSF-1R.

**CSF-1-dependent Differentiation Is Altered in FD-Fms Cells Expressing SLAP-2 SH2*—**FD-Fms cells can be maintained with granulocyte macrophage-colony stimulating factor or IL-3 as macrophage precursor cells with a blast-like phenotype (29). Long-term culture in the presence of CSF-1 results in differentiation into a more complex macrophage-like morphology, and decreased proliferation. These macrophage-like cells exhibit increased cellular granularity, as measured by side-scatter (SSC) and acquire CD11b, a marker of differentiation not expressed on FD-Fms cells grown in IL-3 (29).

Given that expression of SLAP-2 (SH2*) appears to interfere with CSF-1R down-regulation we tested whether this effect would potentiate CSF-1-mediated differentiation of FD-Fms cells. Growth of the different cell lines (HSC, WT SLAP-2, G2A, and SH2*) in IL-3 showed no significant changes in differentiation (Fig. 6A and data not shown). After 3 days of culture in CSF-1, WT SLAP-2 expression slightly decreased CSF-1-mediated differentiation suggesting that SLAP-2 antagonizes the action of CSF-1 (data not shown). However, in agreement with our model, cells expressing SLAP-2 SH2* exhibited a dramatic enhancement in the percentage of cells that differentiated in response to CSF-1, as was measured by SSC and acquisition of CD11b. The percentage of SSC cells was 5–17% greater for the SLAP-2 SH2*-expressing cell line in comparison to the HSC control (Fig. 6A). CD11b acquisition was consistent with the increase in SSC cells such that the percentage of cells that acquired CD11b after growth in CSF-1 was 12–35% greater for the SLAP-2 SH2* cell line compared with the HSC.
control (Fig. 6B). The non-myristoylated SLAP-2 mutant (G2A) showed similar percentages of SSC<sup>high</sup> and CD11b-positive cells as the HSC control cell line. These results indicate that interfering with SLAP-2 function potentiates CSF-1 signaling and suggest therefore that SLAP-2 acts as a negative regulator of CSF-1R.

**SLAP-2 Affects CSF-1R Signaling**—To address which CSF-1-dependent signaling events might be affected, cells expressing either WT or the G2A and SH2<sup>*</sup> mutant forms of SLAP-2 were stimulated with CSF-1. Cell lysates were immunoblotted using phospho-specific antibodies to assess differences in the phosphorylation of several known downstream CSF-1R effectors. No changes in the level of activated ERK or AKT was detected (data not shown), suggesting that the changes evoked by SLAP-2 expression were not due to alterations of the activity of these kinases.

CSF-1R-mediated activation of phospholipase Cγ2 causes generation of diacyl glycerol, which in turn, leads to the plasma membrane translocation and activation of PKC family members (4, 31). Although several PKC isoforms have been demonstrated to be involved in macrophage differentiation, PKC-δ was shown to be activated upon CSF-1 stimulation in FD-Fms cells (32–34). Given that SLAP-2 appears to interfere with CSF-1-mediated differentiation, we looked at the ability of PKC-δ to translocate to the membrane fraction upon CSF-1 stimulation. Membrane fractions of unstimulated or CSF-1-stimulated cells expressing WT, SH2<sup>*</sup>, or G2A forms of SLAP-2 were analyzed by immunoblotting. A decrease in the amount of PKC-δ found in the membrane fraction after CSF-1 stimulation was observed in cells expressing WT SLAP-2 compared with cells containing vector alone (HSC). In contrast, the cell line expressing SLAP-2 SH2<sup>*</sup> showed an increase in PKC-δ in the membrane at 1 and 2 h after CSF-1 stimulation (at 4 °C) compared with both HSC and G2A cell lines (Fig. 7A). Activation of STAT3 has also been shown to occur upon CSF-1 stimulation of FD-Fms cells (35, 36) and suggested to play a role in macrophage differentiation (37). Therefore, we examined the activation of STAT3 with anti-phospho-STAT3 antibodies, after stimulation with CSF-1. Indeed, overexpression of SLAP-2 SH2<sup>*</sup> increased the phosphorylation of STAT3 proteins compared with control cells or cells expressing SLAP-2 WT and G2A (Fig. 7B). Together these observations indicate that SLAP-2 functions to limit the activation of specific CSF-1 response effector pathways.

**DISCUSSION**

Down-regulation of activated RTKs is a key process in terminating signaling from these receptors. As described for other RTKs, the RING-finger protein c-Cbl is involved in CSF-1R internalization and degradation (11). Here we provide evidence that implicates the adaptor protein SLAP-2 in this process. Our data demonstrate a role for SLAP-2 in c-Cbl-dependent down-regulation of CSF-1R.
SLAP-2 is reported to be expressed in bone marrow derived macrophages and more recently has been implicated in CSF-1 signaling. Manes and colleagues report the up-regulation of SLAP-2 during differentiation of mouse bone marrow into primary bone marrow-derived macrophages in response to CSF-1 suggesting that SLAP-2 may be an important regulator in this biological process (28). Similar to our observation, this group also reports an association of SLAP-2 with the CSF-1R thus implying a role in CSF-1R signaling. Our results suggest that the interactions between SLAP-2, c-Cbl, and the CSF-1R in primary bone marrow-derived macrophages may be important for CSF-1R down-regulation thereby limiting the duration of signaling from the CSF-1R in myeloid progenitors.

Our data show that expression of a dominant negative form of SLAP-2 in FD-Fms cells dramatically reduces c-Cbl binding to CSF-1R after stimulation and inhibits ubiquitination, internalization, and post endocytic events leading to CSF-1R degradation. SLAP-2 has previously been shown to be a negative regulator downstream of activated T-cell (18) and B-cell antigen receptors (19). Indeed, overexpression of a dominant negative form of SLAP-2 in Jurkat cells potentiates TCR signaling (17). Similarly we have shown that interfering with SLAP-2 function delays internalization and down-regulation of activated receptors, prolongs CSF-1R signaling, and enhances CSF-1-mediated differentiation of FD-Fms cells.

Because the dominant negative SLAP-2 mutant interacts with c-Cbl and not with the activated receptor, it may act by sequestering c-Cbl away from stimulated receptors or endocytic proteins and thus impair c-Cbl function. We propose that, upon ligand stimulation of CSF-1R, SLAP-2 plays a role in recruiting c-Cbl to the site of activated receptors at the plasma membrane, or in an endosomal compartment thus facilitating receptor ubiquitination and receptor endocytosis. Recently, a closely related family member, SLAP, has been characterized and shown to interact with c-Cbl and TCR complexes in an intracellular compartment, after the initial step of vesicle internalization (21). Similarly, our results may reflect a role for SLAP-2 in modifying a post endocytic step that requires c-Cbl and ubiquitination, because interference with SLAP-2 function disrupted CSF-1R down-regulation. Further studies will determine with which step of endocytosis SLAP-2 SH2* interferes with.

**FIGURE 6.** SLAP-2 SH2* potentiates CSF-1R-mediated differentiation in long term cultures. A, FD-Fms cells stably transduced with either empty vector (HSC), a non-myristoylated mutant (G2A), or a mutant with a non-functional SH2 domain (SH2*) were cultured in the presence of WEHI cell supernatant (IL-3) or CSF-1. Forward scatter (FSC) and side scatter (SSC) were measured on day 3. Scatter plots for each condition show FSC on the y-axis and SSC on the x-axis. A gate was selected to determine the percentage of SSC high cells. B, the percentage of CD11b-positive cells was determined in the cell lines grown for 3 days in the presence of WEHI supernatant (IL-3) or CSF-1 by labeling the cells with PE-conjugated anti-CD11b antibodies. A similar trend was observed for both SSC high and CD11b-positive cells in three independent experiments.

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Our observations reveal differences between the behavior of SLAP-2 adaptor proteins within the context of antigen signaling in lymphocytes and RTK signaling in myeloid cells. For example, in vitro and in vivo binding experiments showed that SLAP-2 associates with the CSF-1 receptor independent of ligand activation, whereas SLAP-2 inducibly associates with activated components of the CD3 complex (18). These observations suggest that SLAP-2 is coupled to RTK signaling in a manner distinct from antigen receptors and that the SLAP-2/CSF-1R association may involve unique mode of binding, because SLAP-2 binding to CSF-1R is SH2-dependent but appears to be phosphorylation independent. Furthermore, tyrosine phosphorylation of SLAP-2 following CSF-1R stimulation, which has not been observed following activation of the TCR, may have as yet unidentified effects on SLAP-2 function (18). Further investigation is needed to determine the details of the SLAP-2 interaction with the CSF-1R, as well as the site(s) of SLAP-2 tyrosine phosphorylation and their role in SLAP-2 function.

These studies also indicate that stable overexpression of SLAP-2 G2A, which lacks the amino-terminal myristoylation signal, had minimal effect on CSF-1R-mediated responses. This suggests that myristoylation of SLAP-2 is essential for its activity. Myristoylation of proteins is known to facilitate association with membrane compartments and incorporation into lipid rafts (38). Indeed, previous work indicates that the SLAP-2 myristoylation site is essential for association with the plasma membrane (17). Future studies are required to establish that SLAP-2 is, in fact, myristoylated in vivo and the role of this modification in membrane targeting of SLAP-2.

Previous studies in lymphocytes have also demonstrated a robust inhibitory effect of SLAP-2 overexpression on TCR sig-
naling and surface CD3 levels, whereas in this study expression of wild-type SLAP-2 had very modest effects on signaling and CSF-1R levels. This may reflect differences in the role of SLAP-2 and c-Cbl in regulation of these distinct classes of receptors, or it may be an indication that the myeloid cells used in this study have levels of SLAP-2 with respect to CSF-1 regulation that are already saturating. Furthermore, because SLAP is also expressed in FD-Fms cells,5 we cannot rule out SLAP also playing a role in CSF-1R regulation. Indeed, SLAP and SLAP-2 have both been demonstrated to function as antagonists of TCR signaling, although each couples to the TCR via a distinct mechanism. Therefore, it is possible the effects observed upon expression of the dominant negative SLAP-2 mutant may in fact be due to dominant inhibitory effects on both SLAP and SLAP-2 function. Further analysis of myeloid lineage cells from SLAP- and SLAP-2-deficient mice should reveal the redundancies and specific functions of these two adaptors.

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