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Regulation of Colony-Stimulating Factor 1 Receptor Signaling by the SH2 Domain-Containing Tyrosine Phosphatase SHPTP1

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SHPTP1 (PTP1C, HCP, SHP) is an SH2 domain-containing tyrosine phosphatase expressed predominantly in hematopoietic cells. A frameshift mutation in the SHPTP1 gene causes the *motheaten* (*me*/*me*) mouse. These mice are essentially SHPTP1 null and display multiple hematopoietic abnormalities, most prominently hyperproliferation and inappropriate activation of granulocytes and macrophages. The *me*/*me* phenotype suggests that SHPTP1 negatively regulates macrophage proliferative pathways. Using primary bone marrow-derived macrophages from *me*/*me* mice and normal littermates, we examined the role of SHPTP1 in regulating signaling by the major macrophage mitogen colony-stimulating factor 1 (CSF-1) (also known as macrophage colony-stimulating factor). Macrophages from *me*/*me* mice hyperproliferate in response to CSF-1. In the absence of SHPTP1, the CSF-1 receptor (CSF-1R) is hyperphosphorylated upon CSF-1 stimulation, suggesting that SHPTP1 dephosphorylates the CSF-1R. At least some CSF-1R-associated proteins also are hyperactivated. SHPTP1 is associated constitutively, via its SH2 domains, with an unidentified 130-kDa phosphotyrosyl protein (P130). P130 and SHPTP1 are further tyrosyl phosphorylated upon CSF-1 stimulation. Tyrosyl-phosphorylated SHPTP1 binds to Grb2 via the Grb2 SH2 domain. Moreover, in *me*/*me* macrophages, Grb2 is associated, via its SH3 domains, with several tyrosyl phosphoproteins. These proteins are hyperphosphorylated on tyrosyl residues in *me*/*me* macrophages, suggesting that Grb2 may recruit substrates for SHPTP1. Our results indicate that SHPTP1 is a critical negative regulator of CSF-1 signaling in vivo and suggest a potential new function for Grb2.

Colony-stimulating factor 1 (CSF-1), also known as macrophage colony-stimulating factor (MCSF), is the major growth factor controlling the proliferation and differentiation of murine macrophages in cell culture and in vivo (46). CSF-1 signaling is mediated by the CSF-1 receptor (CSF-1R), which is encoded by c-*fms*, the cellular homolog of the v-*fms* oncogene (40). CSF-1R is a receptor tyrosine kinase belonging to the platelet-derived growth factor receptor (PDGFR) subfamily. This group of growth factor receptors is distinguished by the presence of five tandem immunoglobulin-like loops in the extracellular domain and a cytoplasmic protein-tyrosine kinase (PTK) domain interrupted by a kinase insert region (38).

The initial steps in CSF-1R signal transduction are understood reasonably well. Upon CSF-1 binding, the CSF-1R dimerizes and the tyrosine kinase domain is activated, resulting in receptor transphosphorylation (4, 19, 30). Four CSF-1R tyrosine phosphorylation sites have been identified (35, 48, 53). Previous studies established that the SH2 domain-containing proteins Grb2 and phosphatidylinositol 3′-kinase (PI-3 kinase) bind to two of these phosphotyrosyl residues (35, 54). Recently, it was reported that Shc, Grb2, and an unidentified 150-kDa tyrosyl-phosphorylated protein form complexes with the tyrosyl-phosphorylated CSF-1R in the murine myeloid progenitor cell line FDC-P1 (20). However, the precise functions of these various signaling complexes and how these initial events are coupled to downstream signal transduction pathways are not known.

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Likewise, little is known about how CSF-1R signaling is negatively regulated. The CSF-1R is rapidly and efficiently internalized following CSF-1 binding, but the importance of receptor internalization in signal termination is not clear (15, 19, 30). Tyrosyl dephosphorylation of the CSF-1R and/or downstream targets also could be important for negative regulation. Although specific protein-tyrosine phosphatases (PTPs) involved in regulating CSF-1R signaling have not been identified, SHPTP1 (PTP1C, HCP, SHP), a nontransmembrane SH2 domain-containing PTP expressed predominantly in hematopoietic cells (25, 34, 39, 57), is an attractive candidate for several reasons.

Biochemical studies have implicated SHPTP1 in many hematopoietic signaling pathways. For example, it was reported that the N-terminal SH2 domain of SHPTP1 directs ligand-induced association with the tyrosyl-phosphorylated interleukin 3R (IL-3R) β chain in the myeloid cell line DA-3 and that reduction in SHPTP1 levels upon expression of antisense RNA led to a slight increase in growth rate in response to IL-3 (59). SHPTP1 stably associates with the activated erythropoietin receptor (17, 60) and probably acts to terminate erythropoietin signaling by inactivating JAK2 (17). Cells expressing mutant erythropoietin receptors unable to bind SHPTP1 show sustained JAK2 activation (17), and insect cell coexpression studies indicate that SHPTP1 efficiently dephosphorylates JAK2 (21). Recently, we have found that SHPTP1 controls the tyrosyl phosphorylation of JAK1 in alpha interferon signaling (10), which suggests that dephosphorylation of receptor-associated Janus family kinases may be a general mechanism by which SHPTP1 negatively regulates cytokine receptors. SHPTP1 negatively regulates the B-cell antigen receptor (8, 9, 12, 32), although its direct targets in this pathway remain undefined. Previous studies also implicated SHPTP1 in regulating RTK pathways. SHPTP1 associates with c-Kit and becomes

tyrosyl phosphorylated upon stem cell factor stimulation of Mo-7e cells (58). In addition, two groups have reported that SHPTP1 becomes tyrosyl phosphorylated following CSF-1 stimulation of the macrophage cell line BAC1.2F5 (56, 58). Association between SHPTP1 and the CSF-1R was not observed, and no other SHPTP1-associated proteins were identified. Despite these indications that SHPTP1 might help regulate CSF-1R signaling, its precise role has not been established.

Genetic evidence also suggests that SHPTP1 is an essential negative regulator of hematopoietic cell signal transduction. Homozygous mutations in the SHPTP1 gene cause *motheaten* (*me*/*me*) and *motheaten viable* (me^v/me^v) mice, respectively (43, 49). These two alleles generate phenotypes that are qualitatively similar but of different severities. Because of an early frameshift mutation, *me*/*me* mice are essentially SHPTP1 null. The me^v/me^v mutation leads to SHPTP1 protein products with markedly decreased PTP activity (43). Although *me*/*me* mice survive for only 2 to 3 weeks, me^v/me^v mice live for a few months (42); presumably, residual PTP activity accounts for the increased viability of me^v/me^v mice.

motheaten mice display multiple defects in hematopoietic cells, which result in immunodeficiency, systemic autoimmunity, and premature death (41). Most prominent among these defects are hyperproliferation and inappropriate activation of myeloid cells. The moth-eaten appearance of their coats is due to sterile subcutaneous accumulations of macrophages and granulocytes; similar accumulations in their lungs cause fatal pneumonitis. These mice also display lymphoid and erythroid abnormalities, some of which may be secondary consequences of the myeloid abnormalities (41). A previous study argued that macrophages from *me*/*me* mice proliferate in the absence of CSF-1 (26). Others have reported enhanced levels of tyrosine phosphorylation in primary macrophages from *me*/*me* mice grown in the continuous presence of CSF-1 (43). Comparison of signal transduction events in normal and *motheaten* mice allows the study of the in vivo role of SHPTP1 in hematopoietic signaling pathways. We have examined the role of SHPTP1 in CSF-1R signaling, using bone marrow-derived macrophages (BMDM) from *me*/*me* mice and their normal littermates. We found that macrophages from *me*/*me* mice are hyperresponsive to CSF-1, although they retain an absolute requirement for CSF-1 for both viability and proliferation. Following CSF-1 stimulation, the CSF-1R is hyperphosphorylated, and at least some of its associated proteins are hyperactivated in *me*/*me* macrophages. We confirmed that, as reported previously, SHPTP1 becomes tyrosyl phosphorylated following CSF-1 stimulation and does not associate stably with the CSF-1R. However, we found that, via its SH2 domains, SHPTP1 is stably associated with an as-yet-unidentified 130-kDa phosphotyrosyl protein. In addition, we have found that, via its SH2 domain, the adapter Grb2 binds to tyrosyl-phosphorylated SHPTP1 upon CSF-1 stimulation. The purpose of this association appears to be recruitment of substrates for SHPTP1. Our results indicate that SHPTP1 is a critical negative regulator of CSF-1 signaling in vivo and suggest a possible new function for Grb2.

MATERIALS AND METHODS

Cells. BMDM were prepared from 2-week-old C3HeB/FeJle-a/a-*me*/*me* mice or their unaffected littermates by a modification of previously described procedures (50). Briefly, bone marrow cells extruded from femurs were incubated for 24 h in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal bovine serum and 20% L-cell conditioned medium (L-CM) as a source of CSF-1. On day 1, nonadherent cells were collected and seeded on 100-mm-diameter plates at 1.5×10^5 cells per ml in DMEM plus 20% L-CM. On day 6, adherent BMDM were deprived of CSF-1 for 24 h before being stimulated with various amounts of human CSF-1 (Genetics Institute) for the indicated times. For proliferation studies, on day 1, nonadherent BMDM were seeded on 35-mm-diameter plates at 3×10^4 to 4×10^4 cells per plate in 2 ml of DMEM with 20% L-CM. On day 4, nonadherent cells were removed, and adherent cells were washed with Hanks' balanced salt solution and incubated in the presence of the indicated amounts of CSF-1. Cell counts were performed in duplicate every 24 h. Plates were washed with phosphate-buffered saline (PBS) and incubated with 1 ml of 0.005% (wt/vol) Zwittergent in PBS for 5 min. Cells were removed by pipetting and counted with a Coulter Counter (Coulter Electronics). In some experiments, the macrophage cell line BAC1.2F5 (28) was used. BAC1.2F5 cells were maintained in RPMI medium supplemented with 10% fetal bovine serum plus 20% L-CM. BAC1.2F5 cells were deprived of CSF-1 for 24 h before being stimulated with 3,000 U of CSF-1 per ml for 10 min.

Antibodies and GST fusion proteins. Affinity-purified antibodies directed against the N terminus (NTM antibodies) and the C terminus (CTM antibodies) of SHPTP1 recognize nonoverlapping regions of SHPTP1. The NTM anti-SHPTP1 antibodies have been described previously (23). Briefly, NTM antibodies were raised against a glutathione *S*-transferase (GST)-SHPTP1 full-length fusion protein but then depleted through a GST-CTM SHPTP1 affinity column. The C-terminal anti-SHPTP1 antibodies were raised in rabbits by immunization with GST-CTM, a GST fusion protein containing the C-terminal 110 amino acids of SHPTP1 (from the *Ava*II site to the *Eco*RI site). CTM antisera were affinity purified on a GST-CTM fusion protein column. Affinity purification was performed as previously described (23). In most immunoprecipitation experiments, matched preimmune immunoglobulins purified on a column of protein A-Sepharose (Pharmacia) were used for controls. Monoclonal anti-SHPTP1 and anti-Grb2 antibodies used for immunoblotting were purchased from Transduction Laboratories. Polyclonal antibodies to the murine CSF-1R and Shc and monoclonal antiphosphotyrosine antibody (4G10) were purchased from Upstate Biotechnology, Inc. Polyclonal antibodies to Grb2 used for immunoprecipitation, polyclonal antibodies to c-Cbl, and monoclonal anti-GST antibodies were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antiphosphotyrosine agarose, used for immunoprecipitation, was purchased from Sigma. Polyclonal antibodies to the p85 subunit of PI-3 kinase were provided by L. Cantley (Beth Israel Hospital, Boston, Mass.). The GST fusion protein containing both the N- and the C-terminal SH2 domains of SHPTP1 [GST-SH2(N+C) protein] has been described elsewhere (17). The GST-Grb2 (full-length) fusion protein was a gift from L. Feig (Tufts Medical School). The GST-Grb2 mutant fusion proteins R86K (with an R-to-K change at position 86), P49L/P206L, and P49L/R86K/P206L were generated by oligonucleotide-directed mutagenesis, using standard techniques (1).

Immunoprecipitations, binding assays, and immunoblotting. Cells were washed in PBS, collected by centrifugation, and lysed in Nonidet P-40 (NP-40) buffer (1% NP-40, 150 mM NaCl, 50 mM Tris [pH 8.0], 1 mM sodium orthovanadate, 10 mM NaF) containing a protease inhibitor cocktail (10 μg of leupeptin per ml, 1 μg of aprotinin per ml, 1 μg of pepstatin A per ml, 1 μg of antipain per ml, and 20 μg of phenylmethylsulfonyl fluoride per ml). Lysates were vortexed and incubated on ice for 15 min at 4°C. For immunoprecipitations, clarified lysates were equalized for protein content by using the bicinchoninic acid assay (Pierce) and then were incubated with the appropriate antibodies and 30 μl of protein A-Sepharose beads for 2 or 3 h at 4°C. Immune complexes were washed four times with 1% NP-40 buffer, resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8 or 10% polyacrylamide), and transferred onto Immobilon (Millipore). For in vitro binding assays, lysates were incubated with glutathione-agarose beads containing 5 μg of GST or the indicated GST fusion protein for 2 h at 4°C. Bound complexes were washed four times and resolved by SDS–8% PAGE. Immunoblots were blocked with 5% bovine serum albumin (BSA) for 1 h, incubated for 1 h with primary antibodies diluted in 5% BSA, and then incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies (Amersham). Immunoblots were visualized by using enhanced chemiluminescence (ECL) reagents (Amersham). For far Western (protein-protein) blots, proteins were transferred to Immobilon, and the blots were blocked with 5% BSA for 1 h. The blots were incubated with various GST-Grb2 fusion proteins (0.5 μg/ml) for 1 h and then subjected to a 1-h incubation with anti-GST monoclonal antibodies (0.1 μg/ml) and a 1-h incubation with horseradish peroxidase-conjugated secondary antibodies. Blots were visualized by using ECL as described above. In some cases, antibodies were stripped from blots by incubation in a buffer containing 100 mM 2-mercaptoethanol–62.5 mM Tris (pH 6.7)–2% SDS for 1 h at 70°C and then reprobed with different antibodies as described above.

RESULTS

BMDM from *me*/*me* mice hyperproliferate in response to CSF-1. The most prominent defects in *motheaten* mice are expansion and inappropriate activation of the myeloid lineage, i.e., granulocytes and macrophages. Since CSF-1 is the major growth factor controlling the proliferation and differentiation of murine macrophages, we examined the proliferative response of *me*/*me* macrophages to CSF-1. As shown in Fig. 1A, BMDM from *me*/*me* mice hyperproliferate in the presence of

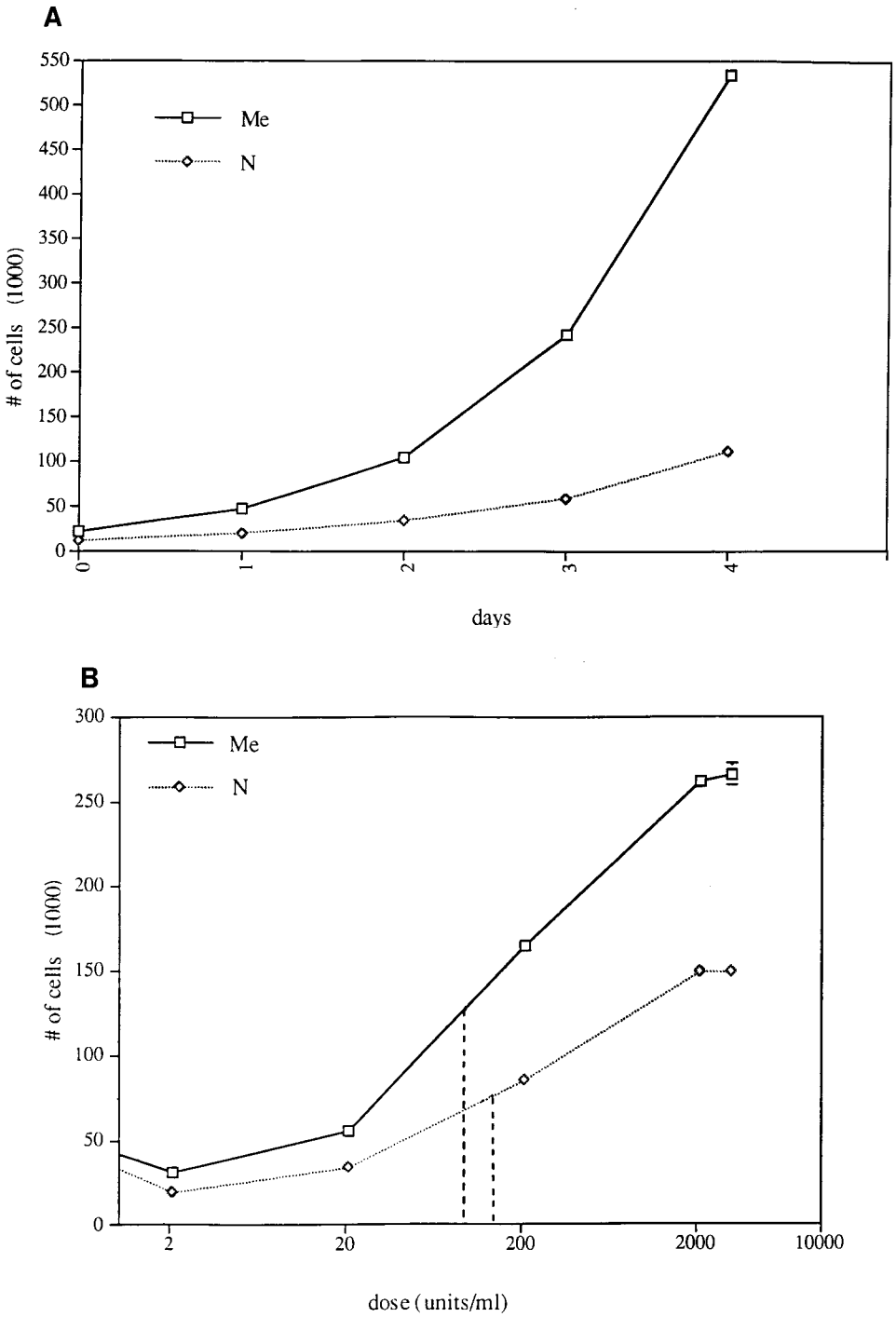

FIG. 1. Hyperproliferation of macrophages from *me/me* mice in response to CSF-1. (A) Growth curves of BMDM from *me/me* mice and their normal littermates in the presence of 2,000 U of CSF-1 per ml. (B) Dose-response curves of BMDM from *me/me* mice and their normal littermates in the presence of indicated concentrations of CSF-1. Proliferation assays were performed as described in Materials and Methods. Error bars represent the standard errors of the means; where no error bars are visible, the error was smaller than the symbol for that point. Me, *me/me* mice; N, normal mice. Dotted lines indicate ED_{50}s.

CSF-1. This hyperproliferation was manifested both by a shortening of the apparent doubling time at the saturating CSF-1 concentration (Fig. 1A) and by a slight leftward shift in the proliferation dose-response curve (Fig. 1B). The decrease in apparent doubling time in these primary cultures could be due to a shortening of the cell cycle, a lower cell death rate, or a decrease in the proportion of cells undergoing differentiation (see Discussion). The leftward shift in the dose-response curve suggests that the CSF-1R signaling pathway may be hypersensitive in *me/me* mice. Although *me/me* BMDM proliferated in response to a lower dose of CSF-1 (10 U/ml) compared with normal macrophages, we found no evidence of proliferation or

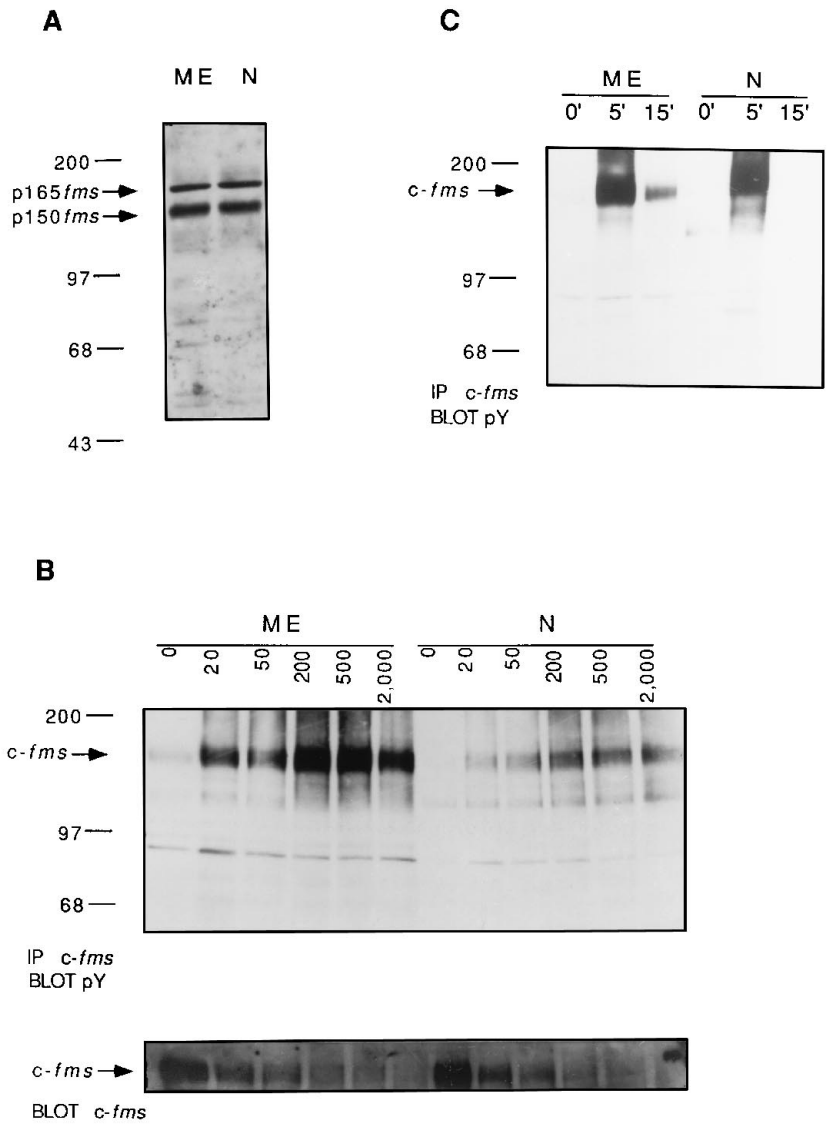

FIG. 2. The CSF-1R is hyperphosphorylated in *me*/*me* macrophages. (A) CSF-1R levels are similar in *me*/*me* (ME) and normal (N) macrophages. BMDM from *me*/*me* and normal mice were lysed and clarified. Total cell lysates (20 μg) were resolved by SDS-PAGE and immunoblotted with an anti-CSF-1R antiserum. (B) CSF-1R phosphorylation in response to various doses of CSF-1 in normal and *me*/*me* macrophages. BMDM from *me*/*me* mice and their normal littermates were stimulated with the indicated concentrations of CSF-1 (in units per milliliter) for 10 min. Lysates from unstimulated and stimulated cells were incubated with CSF-1R antiserum for 3 h. CSF-1R immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody 4G10 (top panel). The blot was then stripped and reprobed with an anti-CSF-1R antiserum (bottom panel). (C) Time course of CSF-1R tyrosyl phosphorylation in normal and *me*/*me* macrophages. BMDM from *me*/*me* mice and their normal littermates were stimulated with CSF-1 (2,000 U/ml) for 5 or 15 min. CSF-1R immunoprecipitates were prepared and analyzed by antiphosphotyrosine immunoblotting as for panel B. Molecular masses (in kilodaltons) are indicated to the left of the gels. IP, immunoprecipitate.

survival in the absence of CSF-1, as had been reported previously (26).

CSF-1R is hyperresponsive in *me*/*me* macrophages. The hyperproliferation of *me*/*me* BMDM in response to CSF-1 suggested abnormal regulation of the CSF-1R signaling pathway. One possible explanation for such hyperresponsiveness could be overexpression of the CSF-1R in *me*/*me* mice. We immunoblotted equal amounts of total lysates from *me*/*me* and normal macrophages with anti-CSF-1R antiserum; as shown in Fig. 2A, their CSF-1R levels were comparable. In addition, biotinylation experiments revealed that similar amounts of the CSF-1R are expressed on the cell surface in *me*/*me* and normal BMDM (data not shown). Given that their CSF-1R levels are the same, the hyperresponsiveness of *me*/*me* macrophages to CSF-1 suggests that, in the absence of SHPTP1, downstream CSF-1 signaling pathways are dysregulated.

Since the CSF-1R is the initial component of this signaling pathway, we examined CSF-1R tyrosyl phosphorylation in response to CSF-1. The murine CSF-1R is a 165-kDa protein that becomes tyrosine phosphorylated upon CSF-1 stimulation. We stimulated *me*/*me* and normal BMDM for 10 min with a range of CSF-1 doses and compared receptor tyrosyl phosphorylation levels by performing antiphosphotyrosine immunoblots on CSF-1R immunoprecipitates. The CSF-1R was found to be hyperphosphorylated on tyrosine in *me*/*me* macrophages (Fig. 2B, top panel). Reprobing this immunoblot with CSF-1R antibodies revealed that comparable levels of CSF-1R were present in the immunoprecipitates from normal and

me/me macrophages (Fig. 2B, bottom panel). However, at higher doses of CSF-1, CSF-1R immunoprecipitates from normal and *me/me* macrophages contained less CSF-1R than in the absence of CSF-1 or at lower CSF-1 doses; this likely reflects significant levels of receptor endocytosis and/or degradation occurring within the period of CSF-1 stimulation (10 min) (5, 19). There were greater differences in CSF-1R tyrosyl phosphorylation levels at lower doses of CSF-1 than at higher doses. This leftward shift in tyrosyl phosphorylation of the CSF-1R seen in *me/me* macrophages correlates with the leftward shift seen in the proliferation dose-response curve (Fig. 1B). In both normal and *me/me* macrophages, the concentration of CSF-1 required to saturate the biological response (proliferation) was greater than that needed to produce saturating levels of receptor tyrosyl phosphorylation immediately following CSF-1 addition (compare Fig. 1B and 2B). Most likely, this is due to the high turnover rate of CSF-1 in primary macrophage cultures, which has been documented previously (50), combined with the requirement that CSF-1 be present throughout most of G_1 (i.e., until the restriction point) to promote S-phase entry (51). A low but detectable level of CSF-1R tyrosyl phosphorylation in the absence of CSF-1 was observed in *me/me* macrophages (Fig. 2B). The extent of this phosphorylation was variable from experiment to experiment (compare Fig. 2B and C) and may have several possible explanations, including unopposed basal activity of the CSF-1R in the absence of ligand, an inability to fully starve these primary cells for CSF-1 without producing substantial amounts of apoptosis in the cultures, or a low level of autocrine production of CSF-1 (see Discussion). However, consistent with the observation that any CSF-1 produced by *me/me* macrophages must be insufficient to allow survival or to promote proliferation (Fig. 1), it also is insufficient to direct substantial receptor phosphorylation and/or surface modulation (Fig. 2B).

We also analyzed the kinetics of tyrosyl phosphorylation of the CSF-1R at saturating concentrations of CSF-1. Although receptor tyrosyl phosphorylation levels are more comparable in *me/me* and normal macrophages after 5 min of CSF-1 stimulation, by 15 min, CSF-1R tyrosyl phosphorylation is undetectable in normal macrophages, whereas it is sustained in *me/me* macrophages (Fig. 2C). The CSF-1R is no longer phosphorylated on tyrosine in either *me/me* or normal macrophages 30 min following stimulation (data not shown). Thus, the hyperphosphorylation of the CSF-1R observed in the dose-response curve in Fig. 2B (which was obtained after 10 min of CSF-1 stimulation) probably indicates that, in the absence of SHPTP1, the CSF-1R is not appropriately dephosphorylated following CSF-1 addition. The simplest and most likely explanation for these results is that SHPTP1 acts directly on the CSF-1R to catalyze receptor dephosphorylation. However, macrophages clearly have other mechanisms for eventually terminating CSF-1 signaling, since even in *me/me* macrophages, CSF-1R phosphorylation eventually returns to basal levels. These alternative mechanisms could include other PTPs and/or endocytosis (see Discussion).

Downstream targets of the CSF-1R are hyperactivated in *me/me* macrophages. Since CSF-1R tyrosine phosphorylation is enhanced in *me/me* macrophages, we expected that receptor target proteins might also be affected. It has been reported that the p85 subunit of PI-3 kinase and Shc bind to the tyrosyl-phosphorylated CSF-1R; in addition, Shc becomes tyrosyl phosphorylated in response to CSF-1. Reprobing the blot in Fig. 2C with antibodies to p85 revealed that although approximately equal amounts of p85 were recovered in CSF-1R immunoprecipitates from *me/me* and normal macrophages 5 min following CSF-1 stimulation, by 15 min, above-basal-level association of PI-3 kinase with the receptor can be detected only in *me/me* macrophages (Fig. 3A). This is consistent with the kinetics of activation of the CSF-1R seen in Fig. 2C and suggests that the p85 binding site on the CSF-1R remains phosphorylated at this time in *me/me* macrophages. To examine whether the activation of Shc also is altered in *me/me* mice, we stimulated *me/me* and normal macrophages for 10 min and compared Shc tyrosyl phosphorylation levels by antiphosphotyrosine immunoblotting of Shc immunoprecipitates. We did not observe stable association of Shc with the CSF-1R. A previous study by Lioubin et al. (20) indicated that Shc is present in a complex which includes the CSF-1R. However, as shown in Fig. 3B, upon CSF-1 stimulation Shc is hyperphosphorylated on tyrosyl residues in *me/me* macrophages. These results indicate that, consistent with the hyperphosphorylation of the CSF-1R in *me/me* macrophages, at least some downstream CSF-1 targets are hyperactivated in these cells.

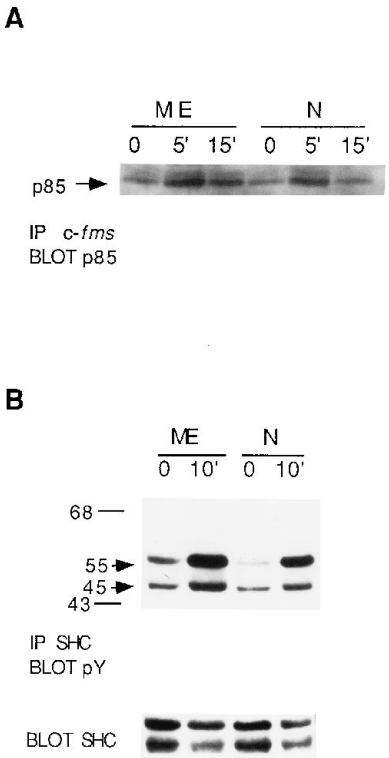

FIG. 3. Downstream CSF-1R targets are hyperactivated in *me/me* macrophages. (A) Association of p85 with the CSF-1R in *me/me* (ME) and normal (N) macrophages. CSF-1R immunoprecipitates prepared as described for Fig. 2C were probed with anti-p85 antibodies. (B) Tyrosyl phosphorylation of Shc in *me/me* and normal macrophages. BMDM from *me/me* and normal mice were stimulated with CSF-1 (2,000 U/ml) for 10 min. Lysates from unstimulated cells (lanes 0) and cells stimulated for 10 min (lanes 10′) were incubated with Shc antiserum for 3 h. The immunoprecipitated proteins were analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine antibody 4G10 (top panel). The blot was then stripped and reprobed with anti-Shc antiserum (bottom panel). Molecular masses (in kilodaltons) are indicated on the left. IP, immunoprecipitate.

SHPTP1 associates with an unidentified 130-kDa protein via its SH2 domains. The above results suggested that the CSF-1R is a target of SHPTP1 following CSF-1 stimulation. To begin to understand the mechanism by which SHPTP1 regulates CSF-1R signaling in more detail, we examined SHPTP1 tyrosyl phosphorylation and associated phosphotyrosyl proteins in starved and CSF-1-stimulated macrophages. For convenience, in some of these experiments, we used BAC1.2F5

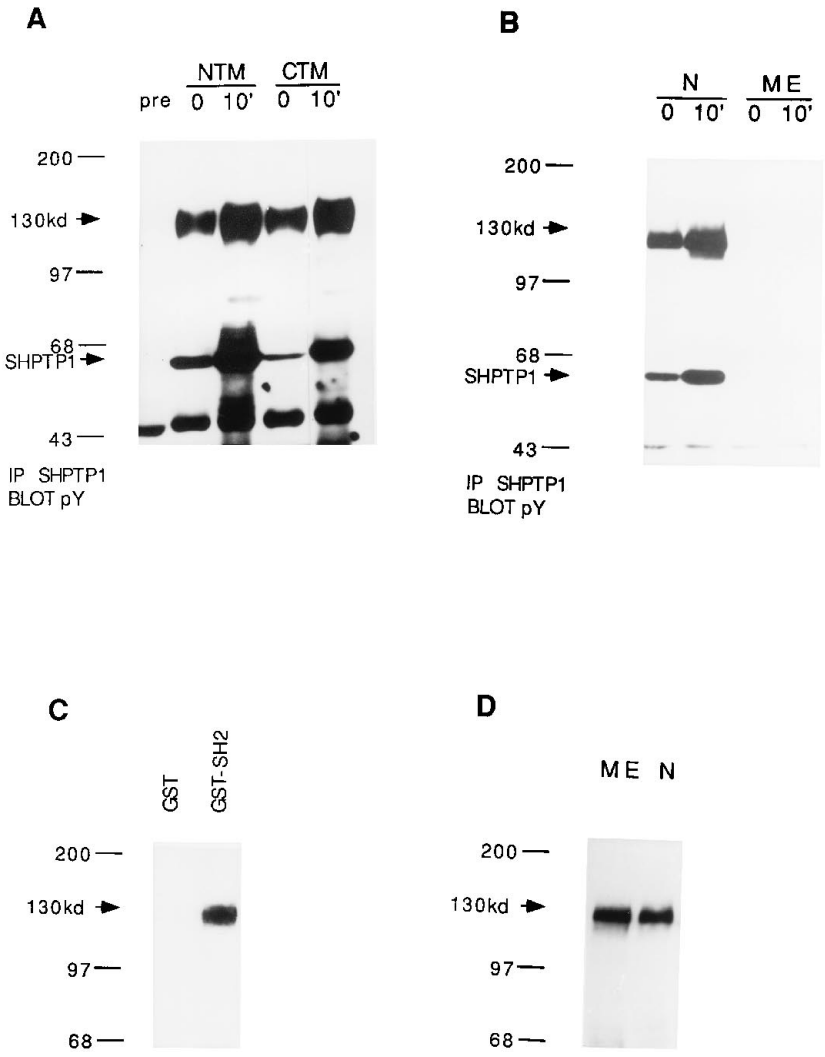

FIG. 4. Association of SHPTP1 with P130. (A) SHPTP1 coimmunoprecipitates with P130. BAC1.2F5 cells were deprived of CSF-1 for 24 h and then stimulated with CSF-1 for 10 min (lanes 10′) or left unstimulated (lanes 0). Lysates from stimulated cells were incubated with matched preimmune immunoglobulins (pre), and lysates from unstimulated and stimulated cells were incubated with affinity-purified NTM or CTM antibodies against SHPTP1 for 2 h. Immunoprecipitates were analyzed by SDS-PAGE and antiphosphotyrosine immunoblotting. (B) Immunoprecipitation of P130 with anti-SHPTP1 antibodies requires the presence of SHPTP1. BMDM from *me*/*me* mice (ME) and their normal littermates (N) were stimulated with CSF-1 (2,000 U/ml) for 10 min. Lysates from unstimulated (lanes 0) and stimulated (lanes 10′) cells were immunoprecipitated with CTM antibodies, and immunoprecipitates were analyzed by antiphosphotyrosine immunoblotting as for panel A. (C) P130 binds to the SH2 domains of SHPTP1. BAC1.2F5 cells were deprived of CSF-1 for 24 h, and cell lysates were incubated with glutathione-agarose beads containing GST or GST-SH2(N+C) for 2 h and then subjected to extensive washing. Bound proteins were separated by SDS-PAGE and subjected to antiphosphotyrosine immunoblotting. (D) P130 is present and tyrosyl phosphorylated in *me*/*me* macrophages. BMDM from *me*/*me* mice and their normal littermates were stimulated with CSF-1 (2,000 U/ml) for 10 min. Lysates from stimulated cells were incubated with GST-SH2(N+C) bound to glutathione agarose and analyzed as for panel C.

cells. BAC1.2F5 is a simian virus 40-immortalized murine macrophage cell line that requires CSF-1 for survival and proliferation and retains many macrophage characteristics (28). All results obtained with BAC1.2F5 cells were verified by using primary macrophages.

Primary macrophages or BAC1.2F5 cells were starved in media without CSF-1, and then were either left unstimulated or stimulated with CSF-1. These cells were lysed and subjected to anti-SHPTP1 immunoprecipitation followed by antiphosphotyrosine immunoblotting (Fig. 4A and B). First, as previously reported by others, we confirmed that SHPTP1 is tyrosyl phosphorylated following CSF-1 stimulation (56, 58). In all of our experiments, there was a detectable, although somewhat variable, level of tyrosyl phosphorylation on SHPTP1 prior to stimulation. Whether this reflects incomplete starvation (as discussed above) or whether other signaling pathways (e.g., from a component of fetal calf serum or from cell adhesion) can contribute to basal tyrosyl phosphorylation of SHPTP1 is not clear. Nevertheless, there was a consistent, marked increase in SHPTP1 tyrosyl phosphorylation in response to CSF-1. Like previous investigators, we did not detect stable association of SHPTP1 and the CSF-1R under our lysis conditions (Fig. 4A and data not shown). However, SHPTP1 was associated constitutively with a 130-kDa phosphotyrosyl protein in both BAC1.2F5 cells (Fig. 4A) and primary macrophages (Fig. 4B). This 130-kDa protein (P130) becomes slightly more phosphorylated upon 10 min of CSF-1 stimulation, suggesting that it may be an intermediate in CSF-1R signaling.

Several lines of evidence indicate that P130 is immunoprecipitated with anti-SHPTP1 antibodies because it forms a com-

plex with SHPTP1 rather than sharing SHPTP1 epitopes. First, P130 coimmunoprecipitated with SHPTP1 when NTM and CTM anti-SHPTP1 antibodies were used (Fig. 4A). These antibodies recognize nonoverlapping determinants, indicating that P130 would have to share multiple epitopes with SHPTP1 to be immunoprecipitated directly by both sets of antibodies. Consistent with the notion that P130 can be immunoprecipitated by anti-SHPTP1 antibodies only if SHPTP1 is present, P130 was not found in anti-SHPTP1 immunoprecipitates from *me/me* macrophages (Fig. 4B). Subsequent studies indicated that P130 is both present and tyrosyl phosphorylated in *me/me* macrophages (Fig. 4D). Finally, P130 associates directly with a GST fusion protein containing both SH2 domains of SHPTP1 (Fig. 4C), indicating that the P130-SHPTP1 interaction is mediated by the SHPTP1 SH2 domains. Consistent with this notion, a GST-SHPTP1 fusion protein containing R-to-K mutations in both SH2 domains failed to bind P130 (data not shown). Interestingly, P130 was by far the most prominent phosphotyrosyl protein that binds to the SHPTP1 SH2 domains in lysates from either unstimulated or stimulated macrophages. This observation allowed us to question whether P130 is a substrate of SHPTP1 in vivo. Lysates from *me/me* and normal macrophages were incubated with the GST-SH2(N+C) fusion protein, and bound proteins were resolved by SDS-PAGE and antiphosphotyrosine immunoblotting. As shown in Fig. 4D, the levels of tyrosyl phosphorylation of P130 in *me/me* and normal macrophages were comparable, suggesting that P130 is not grossly dephosphorylated by SHPTP1 in vivo. Thus far, we have not been able to identify P130 by using a battery of immunological reagents against known phosphotyrosyl proteins (see Discussion).

Grb2 binds to SHPTP1 upon CSF-1 stimulation and may recruit substrates to SHPTP1. Previous studies demonstrated that SHPTP1 is phosphorylated on Y-536 in response to insulin in a lymphoma cell line (52), on Y-564 in a T-cell hybridoma (23), and on both Y-536 and Y-564 in the lymphoma cell line LSTRA (23), which overexpresses Lck. The sequences surrounding both Y-536 (YGNI) and Y-564 (YENV) conform to the consensus recognition sequence (45) for the SH2 domain of Grb 2 (YXNX, where X is any amino acid). Moreover, we have found that Grb2 is stably associated with SHPTP1 in LSTRA cells (22).

Although the site(s) of SHPTP1 tyrosyl phosphorylation in response to CSF-1 has not been reported, we examined whether SHPTP1 could associate with Grb2 in CSF-1-stimulated macrophages. Primary macrophages were stimulated with CSF-1, and SHPTP1 immunoprecipitates were immunoblotted with anti-Grb2 antibodies. As shown in Fig. 5A, Grb2 binds to SHPTP1 upon CSF-1 stimulation. Densitometric scanning of such immunoblots revealed that approximately 2% of Grb2 is associated with SHPTP1 (data not shown). The smaller amount of Grb2 found in SHPTP1 immunoprecipitates from unstimulated macrophages was consistent with the basal levels of tyrosyl-phosphorylated SHPTP1 in these cells (see above and Fig. 4). Conversely, SHPTP1 was coimmunoprecipitated by anti-Grb2 antibodies upon CSF-1 stimulation (Fig. 5B). The apparent discrepancy between the amount of Grb2 that coimmunoprecipitates with SHPTP1 following CSF-1 stimulation and the extent to which SHPTP1 becomes (further) tyrosyl phosphorylated upon CSF-1 addition reflects differences in the extent of stimulation of the primary cells from experiment to experiment (data not shown). To determine the domain(s) of Grb2 that mediates this interaction, BAC1.2F5 cell lysates were incubated with GST fusion proteins containing full-length Grb2 or a GST fusion protein containing a single R-to-K mutation in the SH2 domain (Fig. 6A). As shown

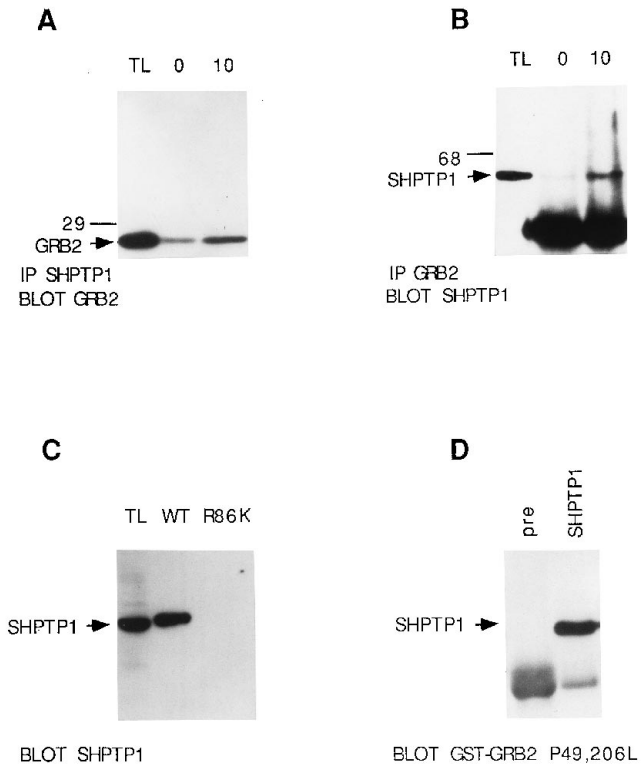

FIG. 5. Grb2 binds to tyrosyl-phosphorylated SHPTP1. (A) Grb2 binds to SHPTP1 upon CSF-1 stimulation. BMDM from normal mice were stimulated with CSF-1 (2,000 U/ml) for 10 min. Total lysates (350 μg) from unstimulated (lane 0) and stimulated (lane 10) cells were incubated with anti-SHPTP1 NTM antibodies. Immunoprecipitates (IP), along with 15 μg of total lysates (TL), were analyzed by SDS-PAGE and immunoblotted with anti-Grb2 antibodies. (B) SHPTP1 binds to Grb2 upon CSF-1 stimulation. Lysates from unstimulated (lane 0) and stimulated (lane 10) cells were immunoprecipitated with anti-Grb2 polyclonal antibodies. Immunoprecipitates, along with 15 μg of TL, were analyzed by SDS-PAGE and immunoblotted with anti-SHPTP1 monoclonal antibodies. (C) SHPTP1-Grb2 interaction requires the Grb2 SH2 domain. BAC1.2F5 cells were stimulated with CSF-1 (3,000 U/ml) for 10 min. Lysates were incubated with glutathione-agarose beads containing the GST-Grb2 wild type (WT) or GST-Grb2 R86K. Bound proteins were separated by SDS-PAGE and immunoblotted with anti-SHPTP1 monoclonal antibodies. (D) The GST-Grb2 SH3 mutant binds directly to SHPTP1. Lysates from CSF-1-stimulated BAC1.2F5 cells were immunoprecipitated with anti-SHPTP1 NTM antibodies or matched preimmune immunoglobulins (pre). Immunoprecipitates were analyzed by SDS-PAGE, and the immunoblot was probed with GST-Grb2 P49L/P206L. Bound GST proteins were analyzed by using anti-GST antibodies as described in Materials and Methods. Molecular masses (in kilodaltons) are indicated to the left of the gels.

in Fig. 5C, the GST-Grb2 full-length protein binds to SHPTP1, whereas inactivation of the Grb2 SH2 domain results in loss of this association, indicating that SHPTP1-Grb2 interaction requires the SH2 domain of Grb2. To determine whether SHPTP1-Grb2 interaction is direct, we performed far Western analysis. Lysates from CSF-1-stimulated BAC1.2F5 cells were immunoprecipitated with anti-SHPTP1 antibodies or preimmune immunoglobulins, and the immunoprecipitates were blotted with a Grb2 fusion protein containing an active SH2 domain but inactive SH3 domains (GST-Grb2 P49L/P206L) (Fig. 6A). As shown in Fig. 5D, SHPTP1 was recognized directly by the Grb2 fusion protein.

Genetic (7, 31, 44) and biochemical (13, 24, 31) evidence implicates Grb2 as a positive signaling molecule, whereas similar types of evidence indicate that SHPTP1 is a negative regulator (see the introduction); thus, their association, at first glance, seems paradoxical. We suspected that the purpose of

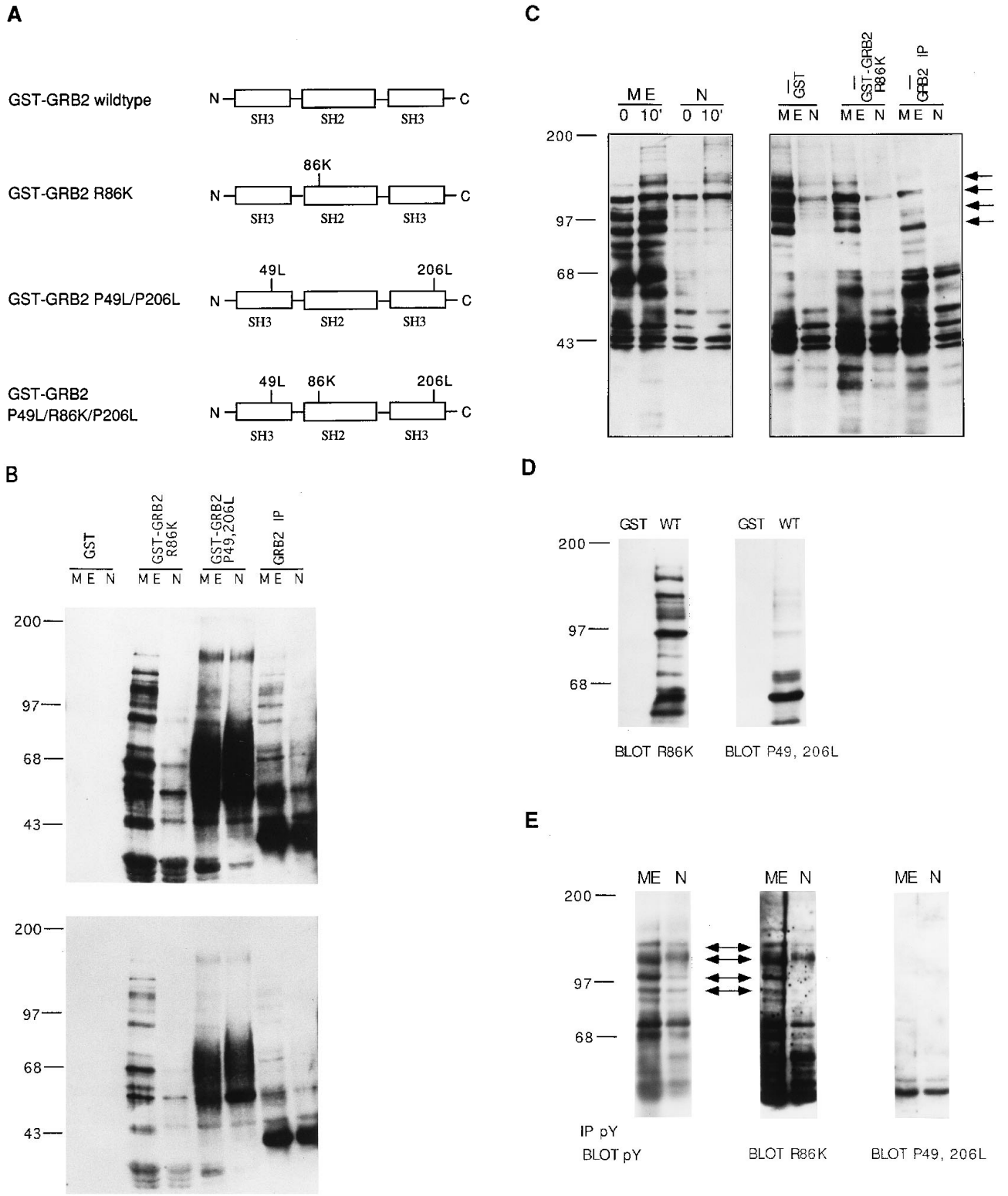

FIG. 6. Grb2 may recruit substrates to SHPTP1. (A) Schematic representation of wild-type and mutant GST-Grb2 fusion proteins. (B) Phosphotyrosyl proteins that are hyperphosphorylated in *me/me* macrophages bind to the Grb2 SH3 domains. BMDM from *me/me* mice (ME) and their normal littermates (N) were stimulated with CSF-1 (2,000 U/ml) for 10 min. Lysates from stimulated cells were incubated with 5 μg of GST, GST-Grb2 R86K, or GST-Grb2 P49L/P206L bound to glutathione-agarose beads, or were immunoprecipitated with anti-Grb2 antibodies. Glutathione-agarose-bound proteins and immunoprecipitates (IP) were separated by SDS-PAGE and subjected to antiphosphotyrosine immunoblotting (top panel, 2-min exposure). The bottom panel is a shorter exposure (20 s) of the same immunoblot to allow better visualization of proteins bound to GST-Grb2 P49L/P206L (P49,206L). (C) Phosphotyrosyl proteins that bind to Grb2 SH3 domains are major hyperphosphorylated proteins in *me/me* macrophages. Total lysates from unstimulated (lanes 0) and stimulated (lanes 10′) *me/me* and normal BMDM were equalized for protein content and then subjected to depletion with the indicated GST protein or with anti-Grb2 antibodies, followed by SDS-PAGE and antiphosphotyrosine immunoblotting. Several proteins that are hyperphosphorylated in *me/me* BMDM (arrows) are depleted with either anti-Grb2 antibodies or GST-Grb2 R86K, which

this association might be to allow Grb2, via its SH3 domains, to recruit substrates to SHPTP1, either before or after CSF-1 stimulation. We favored such a model because in macrophages the SH2 domains of SHPTP1 are, at least to some extent, occupied by P130, although the precise stoichiometry of the P130-SHPTP1 complex is unclear. Moreover, we did not observe any other phosphotyrosyl proteins binding to the SH2 domains of SHPTP1 in CSF-1-stimulated macrophage lysates. Such a hypothesis would predict that (i) Grb2 binds to other proteins besides Sos, via its SH3 domains; (ii) at least some of these proteins are tyrosyl phosphorylated; and (iii) these proteins are hyperphosphorylated in lysates from *me*/*me* macrophages, compared with those of normal macrophages.

To test these predictions, we first used a set of GST-Grb2 fusion proteins in which various domains had been mutated (Fig. 6A) for in vitro binding experiments (Fig. 6B and C). As mentioned above, GST-Grb2 R86K contains a single inactivating mutation in its SH2 domain; this protein is completely unable to bind to phosphotyrosyl proteins via its SH2 domains (24, 37). Thus, specific binding of a tyrosyl-phosphorylated protein to GST-Grb2 R86K would have to be mediated through one or both SH3 domains of Grb2. GST-Grb2 P49L/P206L contains inactivating point mutations in both of its SH3 domains, and GST-Grb2 P49L/R86K/P206L contains point mutations in all three Grb2 functional domains.

The ability of these fusion proteins to associate with phosphotyrosyl proteins in lysates of CSF-1-stimulated macrophages from *me*/*me* and normal mice was assessed. Previously, it was reported that *me*/*me* macrophages grown in the continuous presence of CSF-1 display hyperphosphorylation of a number of phosphotyrosyl proteins (43). Antiphosphotyrosine immunoblots of total lysates from normal and *me*/*me* macrophages removed from CSF-1 for 24 h also revealed hyperphosphorylation of several phosphotyrosyl proteins in *me*/*me* macrophages (Fig. 6C, left panel). Some or all of these proteins could be direct intermediates in CSF-1 signal transduction that are normally dephosphorylated exclusively by SHPTP1. Alternatively, they could be components of signaling pathways that are operative in starved cells (e.g., as part of adhesion-activated or serum-induced signaling pathways) which are preferentially (or exclusively) dephosphorylated by SHPTP1 or proteins that can be ectopically phosphorylated by basal (i.e., unstimulated) levels of one or more cellular PTKs and which normally are not detectable as phosphotyrosyl proteins because of the action of SHPTP1. Notably, however, several of the hyperphosphorylated proteins in starved *me*/*me* macrophages are further phosphorylated by CSF-1 addition in both *me*/*me* and normal macrophages, suggesting that they are CSF-1 signaling intermediates. As shown in Fig. 6B, multiple phosphotyrosyl proteins from lysates from CSF-1-stimulated *me*/*me* macrophages bound to the GST-Grb2 R86K fusion protein, which has active SH3 domains but an inactive SH2 domain. Similar-size proteins in lysates from CSF-1-stimulated normal macrophages bound to this fusion protein, although at substantially lower levels. These results argue that a set of proteins that are hyperphosphorylated in *me*/*me* macrophages, and which therefore represent potential SHPTP1 substrates (see Discussion), are capable of binding to the SH3 domains of Grb2. Consistent with this interpretation, the GST-Grb2 P49L/P206L fusion protein, which contains an active SH2 domain but inactive SH3 domains, associates with a distinct set of phosphotyrosyl proteins (Fig. 6B). No significant binding of tyrosyl-phosphorylated proteins from either *me*/*me* or normal macrophages to either GST alone (Fig. 6B) or to the GST-Grb2 P49L/R86K/P206L fusion protein, in which all three Grb2 functional domains have been inactivated, was observed (data not shown).

These results were consistent with the idea that several proteins that are hyperphosphorylated on tyrosyl residues in *me*/*me* macrophages could associate with Grb2 in vitro via the Grb2 SH3 domains. To determine whether one or more of these proteins actually do bind to Grb2 in vivo, we subjected Grb2 immunoprecipitates from CSF-1-stimulated normal and *me*/*me* macrophages to antiphosphotyrosine immunoblotting (Fig. 6B). No tyrosyl-phosphorylated proteins were observed in immunoprecipitates when nonimmune rabbit antibodies were used (data not shown; see also Fig. 4). However, several tyrosyl-phosphorylated proteins were observed in Grb2 immunoprecipitates from both *me*/*me* and normal macrophages; moreover, these proteins were hyperphosphorylated in *me*/*me* macrophages, and several comigrated with tyrosyl phosphoproteins bound to the GST-Grb2 R86K fusion protein (Fig. 6B).

Many of the Grb2-binding phosphotyrosyl proteins represent major proteins that are hyperphosphorylated (on tyrosyl residues) in *me*/*me* macrophages, as revealed by depletion experiments (Fig. 6C, right panel). In these studies, CSF-1-stimulated lysates from normal or *me*/*me* macrophages were first incubated with GST, GST-Grb2 R86K, or anti-Grb2 antibodies. The proteins remaining in the supernatant following removal of the GST fusion proteins on glutathione-agarose or the immune complexes with protein A-Sepharose, respectively, were resolved by SDS-PAGE and subjected to antiphosphotyrosine immunoblotting. Preincubation with GST removed few phosphotyrosyl proteins from CSF-1-stimulated lysates (Fig. 6C). However, incubation with either GST-Grb2 R86K or with anti-Grb2 antibodies resulted in substantial, in some cases quantitative, depletion of several hyperphosphorylated species from *me*/*me* macrophages (Fig. 6C). Most of the bands depleted by GST-Grb2 R86K were of the same size as those depleted by anti-Grb2 antibodies.

To further test whether the GST-Grb2 R86K and GST-Grb2 P49L/P206L proteins bind directly to distinct sets of proteins, as suggested by in vitro binding experiments above, lysates from CSF-1-stimulated BAC1.2F5 cells were incubated with GST alone or the GST-Grb2 wild-type fusion protein. Bound proteins were subjected to far Western blotting with either GST-Grb2 R86K or GST-Grb2 P49L/P206L protein. As shown in Fig. 6D, the SH3 domain-active fusion protein GST-Grb2 R86K and the SH2 domain-active fusion protein GST-Grb2 P49L/P206L bind to distinct sets of proteins. The set of proteins that associate directly with the SH3 domains of Grb2 was similar in size to the set observed to bind to the SH3 domains

has an inactive SH2 domain but intact SH3 domains. See text for details. The apparent loss of some proteins in the GST-alone depletion lanes was not reproducible in other experiments. (D) Grb2 SH2 and SH3 domain mutants bind directly to distinct sets of proteins. BAC1.2F5 cells were stimulated with CSF-1 for 10 min. Lysates were incubated with glutathione-agarose beads containing GST alone or GST-Grb2 wild type (WT). Bound proteins were separated by SDS-PAGE, blotted with either GST-Grb2 R86K or GST-Grb2 P49L/P206L, and detected by using anti-GST monoclonal antibodies as described for Fig. 5D. (E) Proteins that bind directly to the SH3 domains of Grb2 are hyperphosphorylated in *me*/*me* macrophages. BMDM from *me*/*me* mice and their normal littermates were stimulated with CSF-1 for 10 min. Lysates were subjected to immunoprecipitation with antiphosphotyrosine agarose. Bound proteins were separated by SDS-PAGE and immunoblotted with antiphosphotyrosine antibody 4G10 (pY). The blot was then stripped and reprobed sequentially with GST-Grb2 R86K and GST-Grb2 P49L/P206L proteins as indicated. Molecular masses (in kilodaltons) are shown to the left of the gels.

of Grb2 in the in vitro binding assays whose results are shown in Fig. 6B, suggesting that several of these hyperphosphorylated proteins interact directly with the SH3 domains of Grb2. To confirm this hypothesis, lysates containing equal amounts of protein from CSF-1-stimulated *me/me* and normal macrophages were incubated with antiphosphotyrosine agarose and bound proteins were immunoblotted with antiphosphotyrosine antibodies. As shown in Fig. 6E (left panel), multiple proteins are hyperphosphorylated in *me/me* macrophages; several of these have molecular weights similar to those of the hyperphosphorylated proteins seen in Fig. 6B and C that bind to the SH3 domains of Grb2. Reprobing of the antiphosphotyrosine blot in Fig. 6E (left panel) with the SH3 domain-active GST-Grb2 R86K protein reveals that these phosphotyrosyl proteins bind directly to the SH3 domains of Grb2. Moreover, the signal in lysates from *me/me* macrophages was more intense than that from normal macrophages (middle panel), consistent with the hyperphosphorylation of these proteins in *me/me* macrophages. Reprobing of the same blot with GST-Grb2 P49L/P206L shows that these hyperphosphorylated proteins do not interact directly with the SH2 domain of Grb2 (right panel), which instead identifies a discrete population of phosphotyrosyl proteins, as expected from Fig. 6D.

These findings have several important implications: (i) GST-Grb2 R86K and the Grb2 antibodies likely recognize the same phosphotyrosyl proteins, since both lead to similar depletion of bands of similar molecular weights; (ii) these phosphotyrosyl proteins interact with Grb2 directly via its SH3 domains both in vivo and in vitro; and (iii) a significant number of proteins that are hyperphosphorylated in *me/me* macrophage lysates, and which therefore represent potential substrates of SHPTP1, share these properties. Together, although other explanations remain possible (see Discussion), our data support the hypothesis that at least one function of SHPTP1 tyrosyl phosphorylation, and consequent Grb2 binding to this tyrosyl phosphorylation site(s), is to deliver substrates to the SHPTP1 catalytic domain.

In macrophages, Grb2 interacts with p85 and c-Cbl via its SH3 domains. A recent study reported that the regulatory subunit (p85) of PI-3 kinase binds to Grb2 in vitro and constitutively in NIH 3T3 cells (55). This association was found to be mediated by the SH3 domains of Grb2 and the proline-rich motifs of p85. Another study reported that Grb2 binds, via its SH3 domains (11, 27, 29), to the proline-rich domain of c-Cbl. Both c-Cbl (47) and p85 (see the introduction) have been found to bind to the CSF-1R upon CSF-1 stimulation. Together, these results suggested a possible mechanism by which SHPTP1 could be indirectly recruited via c-Cbl and/or p85 to the CSF-1R (see Discussion). To determine whether Grb2-p85 and/or Grb2–c-Cbl interactions exist in macrophages, BAC1.2F5 cells were stimulated with CSF-1, and p85 or c-Cbl immunoprecipitates were immunoblotted with anti-Grb2 antibodies. As shown in Fig. 7A, Grb2 binds to p85 and to c-Cbl upon CSF-1 stimulation. To determine whether these interactions are direct and whether they require the SH3 domains of Grb2, lysates from CSF-1-stimulated BAC1.2F5 cells were immunoprecipitated with anti-p85 antibodies or anti-c-Cbl antibodies. Immunoprecipitates were blotted with the SH3 domain-active protein GST-Grb2 R86K. As shown in Fig. 7B, both p85 and c-Cbl interact directly with the SH3 domains of Grb2. Consistent with this finding, in vitro binding experiments using the GST-Grb2 R86K and GST-Grb2 P49L/P206L fusion proteins revealed that only the Grb2 SH3 domains, and not the SH2 domain of Grb2, are required for binding to p85 or c-Cbl in BAC1.2F5 cells (data not shown).

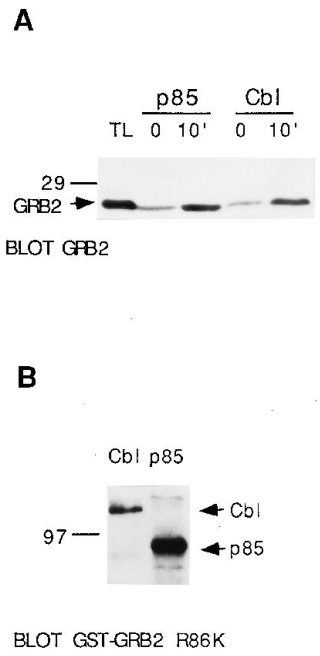

FIG. 7. Grb2 interacts with p85 and c-Cbl via its SH3 domains. (A) Grb2 binds to p85 and c-Cbl upon CSF-1 stimulation. BAC1.2F5 cells were stimulated with CSF-1 for 10 min. Lysates from unstimulated (lanes 0) and stimulated (lanes 10′) cells were immunoprecipitated with anti-p85 or anti-c-Cbl antibodies. Immunoprecipitates were analyzed by anti-Grb2 immunoblotting. (B) Grb2 interacts directly with p85 and c-Cbl via its SH3 domains. Lysates from stimulated BAC1.2F5 cells were immunoprecipitated with anti-p85 or anti-c-Cbl antibodies. Immunoprecipitates were analyzed by SDS-PAGE, blotted with GST-Grb2 R86K, and detected by using anti-GST antibodies. Molecular masses (in kilodaltons) are indicated to the left of the gels.

DISCUSSION

The most prominent hematopoietic abnormalities in *me/me* mice are expansion and inappropriate activation of the myeloid lineage. Since CSF-1 is the major growth factor controlling the proliferation and differentiation of murine macrophages, we suspected that SHPTP1 might be a negative regulator of CSF-1 signaling. This hypothesis was supported by previous biochemical studies, which established that SHPTP1 becomes tyrosyl phosphorylated following CSF-1 stimulation (56, 58). However, little else was known about the mechanism by which SHPTP1 functions in CSF-1R signal transduction. In the current study, we have shown that SHPTP1 is a critical negative regulator of CSF-1 signaling in vivo. BMDM from *me/me* mice display a diminished, although not absent, requirement for CSF-1 to proliferate and, in addition, show enhanced proliferation at all levels of CSF-1. Consistent with these biological observations, in the absence of SHPTP1, the CSF-1R is hyperphosphorylated, and at least some of its associated proteins are hyperactivated following CSF-1 stimulation. In agreement with previous reports, we could not detect an association between SHPTP1 and the CSF-1R under our lysis and immunoprecipitation conditions; instead, we found that in primary macrophages and BAC1.2F5 cells, SHPTP1 is constitutively associated, via its SH2 domain(s), with a previously unreported 130-kDa phosphotyrosyl protein. SHPTP1 is tyrosyl phosphorylated upon CSF-1 stimulation in primary macrophages and BAC1.2F5 cells. Moreover, we found that Grb2, via its SH2 domain, becomes associated with tyrosine phosphorylated SHPTP1 following CSF-1 stimulation. The purpose of this

association may be to recruit to SHPTP1 substrates that bind to the Grb2 SH3 domain(s).

Our results reveal two types of proliferative abnormality in *me*/*me* macrophages (Fig. 1). First, *me*/*me* macrophages proliferate at lower levels of CSF-1 than their normal counterparts (Fig. 1B). However, in repeated experiments, we found no evidence for proliferation in the absence of CSF-1. Without CSF-1, *me*/*me* macrophages, like their normal counterparts, underwent apoptosis (6). The discrepancy between our results and a previous report (26) probably can be explained by differences in experimental design of proliferation assays. The earlier studies used short-term proliferation assays of adherent spleen cells from *me*/*me* mice performed soon after sacrifice, whereas we monitored a more complete growth curve. Factor-producing nonmacrophage cells may have contaminated the short-term adherent splenocyte cultures of the earlier study, thus making it appear as if *me*/*me* macrophages themselves were factor independent. Alternatively, splenic macrophages and BMDM from *me*/*me* mice may have different properties. Our data do not exclude the possibility that low levels of CSF-1 or other growth factors are produced by *me*/*me* macrophages. Indeed, the low but detectable levels of CSF-1R tyrosyl phosphorylation (Fig. 2), as well as the basal hyperphosphorylation of multiple phosphotyrosyl proteins in the absence of CSF-1 (Fig. 6B), could be consistent with autocrine CSF-1 production. However, our data clearly do establish that such factors must be produced at levels that are insufficient to prevent apoptosis, much less promote proliferation. Second, *me*/*me* macrophages show enhanced proliferation at all levels of CSF-1 (Fig. 1B). This results in an apparent decrease in doubling time, from approximately 30 h for normal primary macrophages to approximately 20 h for *me*/*me* macrophages (Fig. 1A).

There are several possible explanations for this 10-h decrease in apparent doubling time. First, the cell cycle of *me*/*me* macrophages could be shorter than that of normal macrophages. Most likely, this would be due to shortening of G_1, although other cell cycle stages could be affected. Alternatively, there could be a lower rate of cell death or a decreased rate of terminal differentiation (to nonproliferative, mature macrophages) in primary cultures of BMDM from *me*/*me* mice; notably, CSF-1 controls both of these processes as well as proliferation. The possibility that *me*/*me* macrophages show a decreased tendency to differentiate, as opposed to proliferating, is of particular interest, as such a finding could imply that SHPTP1 helps to regulate this decision in vivo. Direct measurements of cell cycle lengths and cell death and differentiation rates in normal and *me*/*me* macrophages should resolve these issues.

The hyperresponsiveness of *me*/*me* macrophages to CSF-1 suggested abnormal regulation of the CSF-1R signaling pathway. Obviously, receptor dephosphorylation could be one mechanism by which SHPTP1 down-regulates CSF-1R signaling. Consistent with this notion, the CSF-1R is hyperphosphorylated on tyrosine (Fig. 2B), and its tyrosyl phosphorylation is sustained (Fig. 2C) in *me*/*me* macrophages. Control experiments established that these findings could not be explained by increases in either total CSF-1R levels or CSF-1R surface expression (Fig. 2A and data not shown). The most likely explanation for these results is that the CSF-1R is a direct substrate of SHPTP1. Consistent with this suggestion, a previous study demonstrated that SHPTP1 can dephosphorylate the autophosphorylated CSF-1R in vitro (58). Furthermore, our data suggest that SHPTP1 is the primary mechanism by which CSF-1R signaling is terminated. However, other pathways of inactivation must exist, since the CSF-1R is no longer tyrosyl phosphorylated 30 min after CSF-1 stimulation of either normal or *me*/*me* macrophages (data not shown). It is not clear whether other PTPs dephosphorylate the CSF-1R in the absence of SHPTP1 or whether CSF-1R signaling can also be terminated by other means, such as receptor endocytosis and degradation. In addition, it is not clear whether all sites on the CSF-1R are equivalent targets for SHPTP1. Mutation of certain CSF-1R tyrosyl phosphorylation sites affects the relative ability of the CSF-1R to drive proliferation versus differentiation in FDC-P1 cells (3). If SHPTP1 differentially dephosphorylates CSF-1R sites, the CSF-1R of *me*/*me* macrophages might behave like these mutants, perhaps explaining the apparent decrease in their doubling time (see above). Intriguingly, recent studies suggest that SHPTP1's close relative SHPTP2 may differentially dephosphorylate PDGFR tyrosyl phosphorylation sites in vitro and in vivo (16).

Although SHPTP1 probably dephosphorylates the CSF-1R, we, in agreement with others, did not detect stable CSF-1R–SHPTP1 association. Instead, SHPTP1 constitutively associates, via its SH2 domains, with P130 in both BAC1.2F5 cells and primary macrophages. Like SHPTP1, P130 becomes more phosphorylated (or more P130 becomes associated with SHPTP1) following CSF-1 stimulation. This association implicates P130 as a regulator and/or substrate of SHPTP1. Since the levels of tyrosyl-phosphorylated P130 in normal and *me*/*me* macrophages were comparable after 10 min of CSF-1 stimulation (Fig. 4D), P130 does not appear to be grossly dephosphorylated by SHPTP1. However, we do not exclude the possibility that a subset of P130 phosphorylation sites are SHPTP1 targets.

Elucidation of the function of P130 in CSF-1R signaling and in SHPTP1 regulation will require identification of P130. Unfortunately, P130 does not react with available antibodies against several known signaling molecules of similar molecular weights, including the p130 src substrate (p130CAS), GAP, Jak1, Jak2, Fak, c-Cbl, or the IL3R β chain (data not shown). Interestingly, we find almost no P130-SHPTP1 complex in nonadherent bone marrow cells at any time during establishment of BMDM cultures (6). Either P130 plays a role in adhesion-induced signaling or P130 may be expressed (and/or tyrosyl phosphorylated) only in more-differentiated monocytic cells, or both. Notably, we also find no evidence of P130-SHPTP1 complexes in several hematopoietic suspension cell lines (6). Attempts to obtain sufficient P130 for microsequence analysis are under way.

In addition to its association with P130, SHPTP1 is tyrosyl phosphorylated upon CSF-1 stimulation in both primary macrophages and BAC1.2F5 cells. Like its close relative SHPTP2 (2, 18), tyrosyl-phosphorylated SHPTP1 binds to the adapter Grb2 in vitro and in vivo (Fig. 6). Grb2 is best known as a positive signaling molecule, and its association with SHPTP2 has been proposed to couple Grb2 and Sos to activated receptors, thus contributing to the positive signaling actions of SHPTP2. Since SHPTP1 is clearly a negative regulator in macrophages, it was somewhat surprising to find that Grb2 binds to SHPTP1 via its SH2 domain following CSF-1 stimulation.

Several possible roles for Grb2 binding to SHPTP1 can be envisioned. First, SHPTP1 could activate Ras via the adapter mechanism described above. In this case, SHPTP1 could have both positive and negative signaling functions. Although there is no evidence for a positive signaling role for SHPTP1 in macrophages or other cell types, there exist no functional data to directly support or discredit this possibility. Second, Grb2 binding could stabilize the tyrosyl phosphorylation site(s) on SHPTP1 by acting as a cap to prevent autodephosphorylation. SH2 domains can protect tyrosyl phosphorylation sites from PTP actions in vitro (36), and tyrosyl-phosphorylated SHPTP1 rapidly autodephosphorylates (23, 33). In addition, phosphor-

ylation of SHPTP1 Y-536 has been reported to increase PTP activity fivefold (52). By preventing autodephosphorylation, Grb2 binding might increase the stoichiometry of SHPTP1 tyrosyl phosphorylation, leading to a greater increase in PTP activity. Third, the function of Grb2 binding to SHPTP1 could be to recruit substrates that bind to the Grb2 SH3 domains to tyrosyl-phosphorylated SHPTP1.

Our results are consistent with this substrate recruitment model. In *me*/*me* macrophages, Grb2 is associated with several hyperphosphorylated phosphotyrosyl proteins in vitro and in vivo (Fig. 6B). Using mutants of the various Grb2 functional domains, we established that these interactions are mediated by the SH3 domains of Grb2 (Fig. 6B, D, and E). As revealed by depletion experiments (Fig. 6C) and antiphosphotyrosine immunoprecipitation-far Western blotting experiments (Fig. 6E), several of these are major hyperphosphorylated proteins in *me*/*me* macrophages, consistent with their identification as potential SHPTP1 substrates. These findings suggest that the molecular details by which SHPTP1 negatively regulates CSF-1R signaling may differ significantly from negative regulation of cytokine receptors by SHPTP1, as no clear role for SHPTP1 tyrosyl phosphorylation has been demonstrated in cytokine pathways (10, 17, 59). Thus, distinct SHPTP1 subdomains may be important for its actions in different signaling pathways. We have been unable to detect termolecular complexes in which tyrosyl-phosphorylated SHPTP1 is bound to Grb2, which itself is bound to phosphotyrosyl proteins. This may be explained if, once recruited to tyrosyl-phosphorylated SHPTP1 in normal BMDM, SHPTP1 substrates are rapidly dephosphorylated. Consistent with this notion, in preliminary experiments we found that when normal macrophages were treated with high concentrations of pervanadate, additional tyrosyl-phosphorylated proteins did indeed coimmunoprecipitate with SHPTP1 (data not shown). Several of these proteins have molecular weights similar to those of proteins that bind to the GST-Grb2 SH3 domains and are hyperphosphorylated in *me*/*me* macrophages.

Taken together, our studies suggest a working model for SHPTP1 regulation of the CSF-1R signaling pathway. SHPTP1 is basally associated with P130 via one or both of its SH2 domains. Upon CSF-1 stimulation, the CSF-1R becomes activated and recruits positively signaling SH2 domain-containing proteins such as PI-3 kinase, Grb2, and possibly Shc. In addition, SHPTP1 becomes further tyrosyl phosphorylated. It is not clear whether the CSF-1R or a downstream PTK catalyzes SHPTP1 tyrosyl phosphorylation. If, however, the CSF-1R directly phosphorylates SHPTP1 and/or P130, it is possible that one of these molecules weakly associates with the CSF-1R with an affinity insufficient to survive our immunoprecipitation conditions. Tyrosyl-phosphorylated SHPTP1 then serves to recruit SHPTP1 substrates that bind to the SH3 domains of Grb2. Some (or all) of these substrates may be direct substrates of the CSF-1R, since Grb2 can bind directly (via its SH2 domain) to the CSF-1R.

This model also suggests how, via indirect recruitment by p85 and/or c-Cbl, SHPTP1 could dephosphorylate the CSF-1R without directly binding to it via its SH2 domains. Previous studies indicated that p85 (PI-3 kinase) and c-Cbl could bind to Grb2 via the latter's SH3 domains (11, 27, 29, 55). We have found that similar interactions exist upon CSF-1 stimulation of macrophages (Fig. 7). Upon CSF-1 stimulation, p85, which binds directly to the CSF-1R via its SH2 domains, could recruit tyrosyl-phosphorylated SHPTP1 to the receptor via Grb2. Moreover, following CSF-1 stimulation of the macrophage cell line P388D1, c-Cbl stably binds to the CSF-1R (47); association of c-Cbl with the activated epidermal growth factor receptor has also been reported (14). Thus, c-Cbl also could help recruit SHPTP1 to activated receptor tyrosine kinases, a particularly attractive possibility in view of recent genetic evidence that *Sli-1*, the *Caenorhabditis elegans* homolog of c-Cbl, is a negative regulator of *let-23* signaling (61). A final possibility is that P130 helps target SHPTP1 to the CSF-1R in a manner analogous to that of IRS-1. Our failure to detect indirect association of the CSF-1R with SHPTP1 (via Grb2-p85, Grb2–c-Cbl, or another Grb2–poly-prolyl protein complex) may be due to lower affinity of SH3-polyproline-rich interactions compared with that of SH2-phosphotyrosine interactions and/or to the relatively low stoichiometry of these ternary complexes. However, further studies using reconstitution of *me*/*me* macrophages with appropriate SHPTP1 mutants are required to firmly establish whether the role of SHPTP1 tyrosyl phosphorylation, and Grb2 binding, is solely to recruit substrates and, if so, to elucidate the molecular details of the substrate recruitment model.

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ADDENDUM IN PROOF

While our manuscript was in review, M. Kon-Kozlowski, G. Pani, T. Pawson, and K. Siminovitch reported that Grb2 is associated with SHPTP1 in P815 mastocytoma cells (J. Biol. Chem. **271:**3856–3862, 1996).

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