The Cbl protooncoprotein stimulates CSF-1 receptor multiubiquitination and endocytosis, and attenuates macrophage proliferation

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Colony-stimulating factor-1 (CSF-1) activation of the CSF-1 receptor (CSF-1R) causes Cbl protooncoprotein tyrosine phosphorylation, Cbl–CSF-1R association and their simultaneous multiubiquitination at the plasma membrane. The CSF-1R is then rapidly internalized and degraded, whereas Cbl is deubiquitinated in the cytoplasm without being degraded. We have used primary macrophages from gene-targeted mice to study the role of Cbl. Cbl^{−/−} macrophages form denser colonies and, at limiting CSF-1 concentrations, proliferate faster than Cbl^{+/+} macrophages. Their CSF-1Rs fail to exhibit multiubiquitination and a second wave of tyrosine phosphorylation previously suggested to be involved in preparation of the CSF-1–CSF-1R complex for endocytosis. Consistent with this result, Cbl^{−/−} macrophage cell surface CSF-1–CSF-1R complexes are internalized more slowly, yet are still lysosomally degraded, and the CSF-1 utilization by Cbl^{−/−} macrophages is reduced ~2-fold. Thus, attenuation of proliferation by Cbl is associated with its positive regulation of the coordinated multiubiquitination and endocytosis of the activated CSF-1R, and a reduction in the time that the CSF-1R signals from the cell surface. The results provide a paradigm for studies of the mechanisms underlying Cbl attenuation of proliferative responses induced by ligation of receptor tyrosine kinases.

Keywords: Cbl/cell proliferation/CSF-1 receptor tyrosine kinase/endocytosis/multiubiquitination

Introduction

The Cbl protooncogene product, p120^{c-cbl}, is the cellular homolog of the murine Cas NS-1 retrovirus that induces pro-B, pre-B and myeloid tumors in mice (Langdon *et al.*, 1989a). Cbl is expressed primarily in hematopoietic cells (Langdon *et al.*, 1989a,b) and is tyrosine phosphorylated in responses mediated by both tyrosine kinase and non-tyrosine kinase receptors (reviewed in Miyake *et al.*, 1997; Smit and Borst, 1997). It contains several important protein–protein interaction domains, including a phospho-

tyrosine-binding domain, a ring finger domain, several proline-rich SH3-binding domains and a ubiquitin-associated (UBA) domain. Cbl has been shown to associate in a ligand-dependent manner with several tyrosine kinase receptors, as well as a plethora of other signaling molecules, including Shc, phosphatidylinositol-3 kinase (PI-3K) and intracellular tyrosine kinases (reviewed in Miyake *et al.*, 1997; Smit and Borst, 1997).

Genetic analyses of the *Caenorhabditis elegans* homolog of Cbl, suppressor of lineage defect-1 (SLI-1), indicate that it acts as a negative regulator of vulval induction at the LET-23 [epidermal growth factor (EGF) receptor homolog/SEM5 (Grb2 homolog) step of this Ras pathway (Jongeward *et al.*, 1995; Yoon *et al.*, 1995). The *Drosophila* Cbl homolog D-Cbl has also been reported to have a negative regulatory role in receptor tyrosine kinase signaling (Hime *et al.*, 1997; Meisner *et al.*, 1997). Recent studies have described the Cbl-null (Cbl^{−/−}) mutant phenotype in mice (Murphy *et al.*, 1998). Cbl^{−/−} mice are viable and fertile, but exhibit increased mammary growth. Not unexpectedly, given the high level of expression of Cbl in hematopoietic cells, Cbl^{−/−} mice have increased T-cell receptor expression and tyrosine kinase signaling, lymphoid hyperplasia and primary splenic medullary hematopoiesis. These and other studies (Ota and Samelson, 1997; Ueno *et al.*, 1997) are indicative of a negative regulatory role for Cbl in the control of mammalian cell growth, particularly in the hematopoietic system.

Colony-stimulating factor-1 (CSF-1) regulates the survival, proliferation and differentiation of mononuclear phagocytic cells. The effects of CSF-1 on these target cells are mediated by the CSF-1 receptor (CSF-1R) tyrosine kinase which is encoded by the *c-fms* protooncogene (reviewed in Stanley, 1998). Stimulation of macrophages with CSF-1 causes rapid tyrosine phosphorylation of the CSF-1R and of several, primarily cytoplasmic, proteins (Sengupta *et al.*, 1988). Predominant among these tyrosine-phosphorylated proteins is p120^{c-cbl} (Wang *et al.*, 1996). We have shown that CSF-1 stimulation recruits Cbl to a plasma membrane complex involving the CSF-1R, Shc, PI-3K and Grb2, and that Cbl and the CSF-1R are multiubiquitinated. In contrast to the multiubiquitinated CSF-1R, which is targeted for degradation, multiubiquitinated Cbl dissociates from the CSF-1R, is deubiquitinated and returns to the cytoplasm without degradation (Wang *et al.*, 1996, 1999). These studies raised the possibility that Cbl targets CSF-1R multiubiquitination.

In the present study, we demonstrate that Cbl negatively regulates the macrophage proliferative response to CSF-1. This negative regulation is associated with the targeting of CSF-1R ubiquitination by Cbl and a subsequent increase in the CSF-1R endocytic rate that reduces the time that activated receptors signal from the cell surface.

Results

Transformed characteristics of *Cbl*^{-/-} macrophages

Highly purified primary bone marrow-derived macrophages (BMM), >95% of which express the CSF-1R (reviewed in Stanley, 1990), were obtained from the bone marrow of *Cbl*^{-/-} and *Cbl*^{+/+} mice. When liquid cultures were seeded at low cell density and allowed to form colonies, *Cbl*^{-/-} macrophage colonies were significantly larger and denser (Figure 1C) than *Cbl*^{+/+} colonies (Figure 1A). *Cbl*^{-/-} colonies possessed a dense center due to multiple layers of cells (Figure 1D), in contrast to *Cbl*^{+/+} colonies, which were comprised predominantly of a single layer of cells (Figure 1B). The morphology of *Cbl*^{-/-} and *Cbl*^{+/+} macrophages also differed. In young colonies (Figure 1E and F), *Cbl*^{+/+} cells exhibited the typical bipolar, spindle-shaped morphology of BMM. In contrast, *Cbl*^{-/-} macrophages were much flatter, with lateral as well as leading edge lamellipodia. In addition, *Cbl*^{-/-} macrophages were highly vacuolated compared with *Cbl*^{+/+} macrophages. The greater spreading of *Cbl*^{-/-} macrophages was clearly evident in scanning electron micrographs (Figure 1G and H). CSF-1-starved *Cbl*^{-/-} macrophages were more rounded and retracted than wild-type cells (Figure 1I and J) but, like wild-type cells, were more spread 5 min after readdition of CSF-1 (Figure 1K and L). To investigate the consequences of a lack of Cbl on macrophage growth and survival, BMM were cultured in the presence and absence of high concentrations of CSF-1 (Figure 2A). At low starting cell concentrations, there was no difference in the growth rates of *Cbl*^{+/+} and *Cbl*^{-/-} macrophages and there was no significant difference in their rate of cell death in the absence of CSF-1. However, *Cbl*^{-/-} macrophages reproducibly attained significantly higher cell densities than *Cbl*^{+/+} cells in stationary phase. Thus, *Cbl*^{-/-} macrophages exhibited a significantly more transformed morphology and growth pattern than *Cbl*^{+/+} macrophages.

More efficient proliferation yet unchanged activation of Ras in *Cbl*^{-/-} macrophages

Since loss-of-function mutations of the *C.elegans* Cbl homolog, SLI-1, rescue hypomorphic mutations of *let23* and have no effect on a *let23* wild-type background, we compared the growth of *Cbl*^{+/+} and *Cbl*^{-/-} BMM cultured for 6 days at suboptimal CSF-1 concentrations with daily medium changes (Figure 2B). At all CSF-1 concentrations ≥ 0.6 ng/ml, *Cbl*^{-/-} cells grew significantly faster than *Cbl*^{+/+} cells. At the two highest CSF-1 concentrations, which are not limiting, *Cbl*^{-/-} cells density-arrested at a higher cell concentration than wild-type macrophages, as shown in Figure 2A. The differences in cell density between *Cbl*^{+/+} and *Cbl*^{-/-} cells were not due to a higher apoptotic rate of *Cbl*^{+/+} cells because there was no difference in apoptotic cell number, determined by the TUNEL method (Gavrieli *et al.*, 1992), between *Cbl*^{+/+} and *Cbl*^{-/-} cells cultured at either 6.5 or 442 ng/ml CSF-1, using a positive control of UV-irradiated cells (data not shown). Thus, Cbl attenuates CSF-1-stimulated proliferation of primary macrophages.

At non-limiting CSF-1 concentrations, CSF-1 is consumed at the rate of ~ 14 ng/ 10^5 cells/24 h (Tushinski *et al.*, 1982). To determine the extent of utilization of CSF-1 during the last 24 h of culture in the experiment

shown in Figure 2B, the media from the last feed were collected and assayed for biologically active CSF-1 (Figure 2C). At ≤ 12 ng/ml, the CSF-1 was completely exhausted from all cultures. Thus, despite their consumption of an equivalent amount of CSF-1, *Cbl*^{-/-} cells proliferated more than *Cbl*^{+/+} cells, i.e. they possess greater proliferation signaling efficiency per unit of CSF-1 consumed.

The differences in proliferation and CSF-1 utilization rates between *Cbl*^{-/-} and *Cbl*^{+/+} BMM might be explained by differences in their expression of the CSF-1R or the affinity of their receptors for CSF-1. However, as shown in Figure 3A, both the number and affinity of CSF-1Rs on *Cbl*^{-/-} and *Cbl*^{+/+} BMM, determined by [¹²⁵I]CSF-1 binding (Guilbert and Stanley, 1986), were indistinguishable. Since the Cbl homolog SLI-1 inhibits receptor tyrosine kinase signaling along the Ras pathway in *C.elegans* (Jongeward *et al.*, 1995; Yoon *et al.*, 1995), and signaling via the Ras pathway is important for proliferative responses in several mammalian cell types (reviewed in Lowy *et al.*, 1991), we compared CSF-1-stimulated Ras activation in *Cbl*^{+/+} and *Cbl*^{-/-} cells. There was no significant difference between the two cell types in the GTP loading of Ras at 2 min over a CSF-1 concentration range that gave maximum stimulation of GTP loading (2.5-fold) (Figure 3B) or at 120 ng/ml CSF-1 over a 10 min time course (Figure 3C), indicating that the differences in proliferation efficiency cannot be explained by differences in the extent or duration of Ras activation.

CSF-1-mediated tyrosine phosphorylation is altered in *Cbl*^{-/-} macrophages

Cbl is tyrosine phosphorylated rapidly and associates rapidly with the CSF-1R and several other tyrosine-phosphorylated and adaptor proteins following stimulation of macrophages with CSF-1 (Kanagasundaram *et al.*, 1996; Wang *et al.*, 1996, 1999; Husson *et al.*, 1997). We therefore examined CSF-1-induced tyrosine phosphorylation in *Cbl*^{+/+} and *Cbl*^{-/-} cells. Consistent with their genotype, Cbl was not detected in *Cbl*^{-/-} macrophages by Western blotting with anti-Cbl antibody (Figure 4A, upper panel). Surprisingly, however, given the negative regulatory role of Cbl, stimulation of protein tyrosine phosphorylation of both the CSF-1R and non-CSF-1R proteins was significantly reduced in *Cbl*^{-/-} compared with *Cbl*^{+/+} BMM (Figure 4B, middle panel). However, the tyrosine phosphorylation of both the CSF-1R and several non-CSF-1R proteins was more prolonged (Figure 4B, middle panel), indicative of prolonged activation of the CSF-1R.

Coincident with its multiubiquitination, the CSF-1R exhibits a second wave of tyrosine phosphorylation that is associated temporally with increased serine phosphorylation (Baccarini *et al.*, 1991; Li and Stanley, 1991; Wang *et al.*, 1999) (shown schematically in Figure 9). The two waves of CSF-1R tyrosine phosphorylation can be resolved following stimulation at 37°C, but the kinetic differences are discriminated more easily by stimulating cells at 4°C. At 4°C, the changes in the CSF-1R and protein tyrosine phosphorylation mimic those at 37°C, but are enhanced and occur over an extended time frame (Sengupta *et al.*, 1988; Li and Stanley, 1991). Thus, to determine whether there were obvious qualitative differ-

Fig. 1. Colonial and cell morphology of BMM from $Cbl^{+/+}$ and $Cbl^{-/-}$ mice. (A–D) methylene blue-stained macrophage colonies; (E and F) phase-contrast photomicrographs of growing cells; (G–L) scanning electron micrographs of cells grown with 120 ng/ml CSF-1 (G and H), without CSF-1 for 16 h (I and J) and without CSF-1 for 16 h then with CSF-1 for 5 min (K and L). Bars: (A and C) 2.5 mm; (B and D) 0.5 mm; (E and F) 100 μ m; (G–L) 10 μ m.

Fig. 2. Growth and survival of BMM in the presence and absence of CSF-1. (A) Growth of Cbl^{+/+} (squares) and Cbl^{-/-} (circles) BMM grown with (filled symbols) or without (open symbols) 120 ng/ml CSF-1. Each point represents the cell number and standard deviation determined from triplicate dishes. The insert shows the cell number from days 3–8 plotted on a linear scale (differences significant at asterisked time points; *p < 0.001; **p < 0.01). (B) CSF-1 concentration dependence of growth of Cbl^{+/+} (filled) and Cbl^{-/-} (unfilled) BMM. The horizontal line indicates the starting cell concentration. Differences were significant (*p* < 0.0001) at each concentration. (C) Percentage utilization of CSF-1 from the medium of the cultures in the last day of culture (day 6). The experiments shown in this figure have been repeated with similar results, including the significant differences in cell concentration at days 4–8 in (A).

ences in the phosphorylation of the receptor in Cbl^{+/+} and Cbl^{-/-} cells during the first and second waves of tyrosine phosphorylation, ³²P-labeled BMM were stimulated with CSF-1 at 4°C for 20 min (first wave) or 2 h (second wave) (Li and Stanley, 1991), lysed and CSF-1R immunoprecipitates electrophoresed and autoradiographed. Consistent with the reduction of tyrosine phosphorylation in Cbl^{-/-} versus Cbl^{+/+} BMM, both tyrosine phosphorylation (Figure 5I) and total phosphorylation (Figure 5H) of the CSF-1R was significantly lower in Cbl^{-/-} cells at both 20 min and 2 h of stimulation. The CSF-1R bands (Figure 5H) were subjected to phosphopeptide mapping as described in the Materials and

Fig. 3. Cell surface CSF-1R expression and CSF-1 stimulation of Ras GTP loading in Cbl^{+/+} and Cbl^{-/-} BMM. (A) High affinity binding of [¹²⁵I]CSF-1 to Cbl^{+/+} (filled) and Cbl^{-/-} (open) BMM at 4°C. Additional binding experiments included the determination of the 4°C on-rate constants (*k*_{on}): Cbl^{+/+}, 1.00 × 10⁶/M/s; Cbl^{-/-}, 1.08 × 10⁶/M/s; and the 4°C off-rate constants (*k*_{off}): Cbl^{+/+}, ≤ 2 × 10⁻⁷/s; Cbl^{-/-}, ≤ 2 × 10⁻⁷/s (data not shown). (B) BMM were pre-incubated with [³²P]phosphate and incubated with the indicated concentrations of CSF-1 at 37°C for 2 min. Cell lysates were immunoprecipitated with anti-Ras antibody and GTP and GDP in the immunoprecipitates separated by thin-layer chromatography. The percentage GTP loading [GTP/(GTP+GDP) × 100] was based on quantitation by PhosphorImager. (C) BMM were pre-incubated with [³²P]phosphate and incubated with 120 ng/ml CSF-1 at 37°C for the indicated time, prior to the determination of the percentage Ras GTP loading as in (B). The experiments in (B) and (C) have been repeated with similar results.

methods. The pattern of phosphorylation in Cbl^{+/+} cells stimulated for 20 min (Figure 5A) and for 2 h (Figure 5D) was quite different, the 2 h map containing four phosphopeptides not present in the 20 min map, and the 20 min map containing two peptides not present in the 2 h map (Figure 5G), clearly indicating that the first and second waves of CSF-1R phosphorylation involve different sites. There were also qualitative differences between the maps for Cbl^{+/+} (Figure 5A) and Cbl^{-/-} (Figure 5B) cells stimulated for 20 min (Figure 5C), indicating that Cbl influences the sites phosphorylated during the first wave. In addition, after 2 h of stimulation, one phosphopeptide was detected in Cbl^{+/+} (Figure 5D) and not in Cbl^{-/-} (Figure 5E) cells and one phosphopeptide (1 in Figure 5F) was much more intense and another (2 in Figure 5F) less intense in Cbl^{-/-} cells. These results indicate that the presence of Cbl influences the level of CSF-1R phosphorylation, the sites involved and the duration of CSF-1R tyrosine phosphorylation.

Fig. 4. Time course of CSF-1-induced protein tyrosine phosphorylation in Cbl^{+/+} and Cbl^{-/-} BMM at 37°C. Cells were stimulated with 360 ng/ml CSF-1 at 37°C for the indicated time, lysed with NP-40 lysis buffer and processed as described in Materials and methods. (A) Cell lysates were subjected to gradient SDS-PAGE and Western blotted with anti-Cbl antibody. Each lane contains one-tenth of the lysate of one 100 mm dish of cells. (B) Anti-phosphotyrosine (PY) immunoprecipitates of the NP-40 lysates were subjected to gradient SDS-PAGE and Western blotting with the indicated antibodies. Each lane contains PY proteins of the lysate obtained from one 100 mm dish of cells. Direct anti-PY Western blotting was performed with the membrane from (A) with similar results, but resulted in a much lower sensitivity of detection of PY proteins (data not shown).

CSF-1-induced CSF-1R internalization is delayed in Cbl^{-/-} BMM

Internalized CSF-1-CSF-1R complexes are committed to destruction and there is little or no recycling of the CSF-1R in BMM (Guilbert and Stanley, 1986). CSF-1R Western blots suggested that CSF-1-induced internalization and/or degradation of the CSF-1R is slower in Cbl^{-/-} BMM (data not shown). We therefore compared the initial rate of receptor-mediated CSF-1 uptake by Cbl^{+/+} and Cbl^{-/-} cells at 37°C. Cbl^{-/-} BMM, internalized [¹²⁵I]CSF-1 2–3 times more slowly than Cbl^{+/+} BMM (Figure 6A). Decreased internalization of the CSF-1-CSF-1R complex by Cbl^{-/-} BMM was also supported by analysis of the kinetics of CSF-1R internalization. Immunoprecipitation of CSF-1R from the total cell lysates followed by Western blotting with anti-CSF-1R intracellular domain antibodies showed little apparent change in the level of mature ~165 kDa CSF-1R or the 135 kDa high mannose precursor in either cell type up to 5 min of stimulation with CSF-1 (Figure 6B, left upper panel). As expected, the mature ~165 kDa form but not the ~135 kDa precursor was tyrosine phosphorylated and, consistent with the results shown in Figure 4B, tyrosine phosphorylation of the

165 kDa CSF-1R was less in Cbl^{-/-} than in Cbl^{+/+} cells (Figure 6B, right upper panel). Importantly, at 10 and 30 min of stimulation, fragments of ~105 and ~55 kDa were more prominent and appeared earlier in Cbl^{+/+} cells than in Cbl^{-/-} cells. For example, the level of the ~105 kDa CSF-1R cleavage product in Cbl^{+/+} cells at 10 min of stimulation was equivalent to that seen in Cbl^{-/-} cells after 30 min of stimulation. The ~105 and ~55 kDa CSF-1R fragments contain the CSF-1R cytoplasmic domain as evidenced by their detection both by antibodies to intracellular domain CSF-1R peptides (Figure 6B, left lower panel) and by anti-phosphotyrosine (anti-PY) antibody (Figure 6B, right lower panel).

Cell surface receptors decreased significantly more rapidly in Cbl^{+/+} cells (Figure 6B, left middle panel). This pattern was mimicked in the anti-PY Western blot (Figure 6B, right middle panel) although, as expected, the overall stimulation of tyrosine phosphorylation was less in Cbl^{-/-} cells. Cell surface expression of the tyrosine-phosphorylated receptor was also clearly prolonged in Cbl^{-/-} compared with Cbl^{+/+} cells.

It is difficult to appreciate any differences in the levels of internal receptor from the anti-CSF-1R Western blot of the immunoprecipitated internal CSF-1R fraction (Figure 6B, left lower panel), presumably due to contributions from a significant internal pool of mature receptors (~67% of total mature receptors in unstimulated cells) (Guilbert and Stanley, 1986) and from newly internalized receptors. However, consistent with the more rapid loss of cell surface receptors from Cbl^{+/+} cells, the ~105 kDa CSF-1R degradation fragment appeared earlier and more intensely in Cbl^{+/+} cells (Figure 6B, left lower panel) and the internalized tyrosine-phosphorylated receptors (Figure 6B, right lower panel) disappeared more rapidly in Cbl^{+/+} than in Cbl^{-/-} cells.

These experiments show that Cbl^{+/+} cells internalize and degrade the CSF-1R more rapidly than Cbl^{-/-} cells. Consistent with these observations, Cbl^{-/-} cells consumed less CSF-1 than Cbl^{+/+} cells in long-term cultures in non-limiting CSF-1 concentrations (Figure 6C).

CSF-1-induced CSF-1R multiubiquitination is decreased in Cbl^{-/-} macrophages and not restored by elevation of CSF-1R tyrosine phosphorylation

Cbl itself associates with the CSF-1R and is multiubiquitinated in response to CSF-1 with exactly the same kinetics as the CSF-1R (Wang *et al.*, 1996, 1999). Furthermore, compared with Cbl^{+/+} cells, there was little smearing of the tyrosine-phosphorylated cell surface CSF-1R band recovered from Cbl^{-/-} cells (Figure 6B, right middle panel). We therefore examined CSF-1R multiubiquitination in response to CSF-1 in Cbl^{+/+} and Cbl^{-/-} cells. CSF-1R immunoprecipitates from unstimulated cells and cells stimulated with CSF-1 were subjected to SDS-PAGE and immunoblotting with anti-CSF-1R and anti-ubiquitin antibodies (Figure 7A). CSF-1R multiubiquitination was dramatically reduced and slower to develop in Cbl^{-/-} cells. The small amount of residual CSF-1-induced CSF-1R multiubiquitination observed in Cbl^{-/-} cells may be associated with redundancy due to an effect of the closely related Cbl-b, which is also expressed in macrophages (Keane *et al.*, 1995; Hofmann and Bucher, 1996).

Since CSF-1R tyrosine phosphorylation precedes

Fig. 5. Qualitatively different patterns of CSF-1R phosphorylation in CSF-1-stimulated Cbl^{-/-} and Cbl^{+/+} BMM revealed by two-dimensional phosphopeptide mapping. ³²P-labeled Cbl^{+/+} and Cbl^{-/-} BMM were stimulated with CSF-1 at 4°C for 20 min and 2 h. Anti-CSF-1R immunoprecipitates of the ³²P-labeled CSF-1R were subjected to SDS-PAGE and the ³²P-labeled CSF-1R bands located by autoradiography of the wet gel. The bracketed regions containing these bands (H) were excised and subjected to two-dimensional phosphopeptide analysis. (A, B, D and E) Phosphopeptide maps. (C, F and G) Summary of the data. (C) Comparison of (A) and (B) (filled, shared; hatched, Cbl^{+/+} only; open, Cbl^{-/-} only); (F) comparison of (D) and (E) (filled, shared; hatched, Cbl^{+/+} only); (G) comparison of (A) and (D) (filled, shared; open, 20 min only; hatched, 2 h only); (I) anti-PY Western blot of anti-CSF-1R immunoprecipitates derived from a separate 4°C stimulation experiment for the indicated times. Exposure times: (A and B) 18 days; (D and E) 12 days.

CSF-1R multiubiquitination (Wang *et al.*, 1999), it is possible that the primary effect of the absence of Cbl is to lower CSF-1R tyrosine phosphorylation (Figure 5I), reducing conformational changes in the CSF-1R that lead to its multiubiquitination. We therefore increased CSF-1R tyrosine phosphorylation by pre-incubating cells with the protein tyrosine phosphatase inhibitors iodoacetic acid (IAA) or pervanadate, prior to stimulation with CSF-1 at 4°C (Figure 7B). Pre-incubation with IAA or pervanadate dramatically increased the degree of CSF-1-induced CSF-1R tyrosine phosphorylation in Cbl^{+/+} and Cbl^{-/-} BMM to levels much higher than in untreated control cells. In the presence of pervanadate, multiubiquitination of the CSF-1R was not increased substantially, despite the fact that the levels of CSF-1R tyrosine phosphorylation achieved were similar to those of stimulated wild-type cells. These findings suggest that either the tyrosine phosphorylation of the CSF-1R in Cbl^{+/+} cells is qualitatively different from its phosphorylation in Cbl^{-/-} cells, or that the tyrosine phosphorylation state of the CSF-1R is unimportant for its multiubiquitination.

CSF-1-induced CSF-1R degradation is lysosomal and does not involve the proteasome

The appearance of an ~105 kDa tyrosine-phosphorylated CSF-1R fragment exclusively in the internal fraction at 10 and 30 min (Figure 6B) is consistent with cleavage of part of the extracellular domain after CSF-1-induced internalization of the CSF-1R–ligand complex. To determine whether Cbl-enhanced multiubiquitination of the CSF-1R results in its proteasomal or lysosomal degradation, the fate of the receptor in Cbl^{+/+} and Cbl^{-/-} cells following stimulation with CSF-1 in the presence and absence of both lysosomal (methylamine) and proteasomal (lactacystin) inhibitors was investigated (Figure 8). The loss of the 165 kDa CSF-1R band and the generation of CSF-1R fragments of ~55 kDa were coordinately inhibited in Cbl^{+/+} cells treated with the lysosomal inhibitor methylamine, but were unaffected by inhibitors of the proteasome (Figure 8A, upper panel) (the predominance of ~105 versus ~55 kDa CSF-1R breakdown products appears to be cell density-dependent). Leupeptin, a specific lysosomal protease inhibitor, and chloroquine, a less potent lysosomal

Fig. 6. CSF-1-stimulated internalization of the CSF-1R in Cbl^{+/+} and Cbl^{-/-} BMM at 37°C. (A) [¹²⁵I]CSF-1 internalization. Cells were incubated with 120 ng/ml of mouse [¹²⁵I]CSF-1 in the presence and absence of a 500-fold excess of unlabeled CSF-1 at 37°C and the internalized radioactivity (specific uptake) determined as described in Materials and methods. ■, Cbl^{+/+}; ●, Cbl^{-/-}. Each point represent the mean and standard deviation determined from triplicate dishes. (B) CSF-1R internalization. Cells were deprived of CSF-1 for 16 h and then incubated with 120 ng/ml CSF-1 at 37°C for the indicated times. Following the incubation, cells were immediately washed and either lysed with NP-40 lysis buffer and subjected to CSF-1R immunoprecipitation (Total) or cooled rapidly to 4°C and incubated with anti-CSF-1R antiserum at this temperature for 15 min. The excess unbound anti-CSF-1R was removed by washing, the cells lysed in NP-40 lysis buffer and cell surface CSF-1R immunoprecipitates collected by incubation with protein G-Sepharose and centrifugation (Cell Surface). The internal pool of receptors in the remaining cell surface receptor-depleted lysate was immunoprecipitated with anti-CSF-1R antibodies (Internal). All immunoprecipitates were subjected to SDS-PAGE and Western blotting with anti-CSF-1R antibodies to cytoplasmic domain peptides and with anti-PY. The small degree of tyrosine phosphorylation apparent at zero time in both mutant and wild-type cells may be due to antibody cross-linking during cell surface receptor isolation. (C) Utilization rate of CSF-1 in long-term cultures. Utilization was determined over a 24 h period for cells in both stationary (Experiment 1, 442 ng/ml CSF-1) and log (Experiment 2, 123 ng/ml CSF-1) phase growth. Means ± standard deviations. Differences between Cbl^{+/+} (filled) and Cbl^{-/-} (unfilled) cells are significant ($n = 3$, Student's *t*-test; experiment 1, $p \leq 0.04$; experiment 2, $p \leq 0.02$). Note that compared with Figure 2C, the CSF-1 utilization rate is normalized with respect to cell number.

inhibitor than methylamine (Guilbert *et al.*, 1986), also inhibited CSF-1R degradation, but less effectively. Another proteasomal inhibitor, MG132, also failed to inhibit (data not shown). Similar results were observed with the Cbl^{-/-} cells, in which the degree of receptor degradation was less (Figure 8A, lower panel). The effectiveness of the lactacystin inhibition is apparent from the selective accumulation of ubiquitinated proteins in the lactacystin-treated cells (Figure 8B). Densitometric analysis of the Cbl^{+/+} and untreated Cbl^{-/-} data in Figure 8A are presented in Figure 8C. Thus, despite enhancement of CSF-1R multiubiquitination by Cbl, CSF-1R degradation is lysosomal.

Discussion

In the present study, we have used a Cbl-null mutation to demonstrate that Cbl negatively regulates proliferation

signaling by a receptor tyrosine kinase and provided insight into the mechanisms involved. Previous studies showed that inhibition of the generation of higher molecular weight multiubiquitinated CSF-1Rs with a higher phosphotyrosine content than the 165 kDa species (Figure 9, step 4) resulted in increased cellular protein tyrosine phosphorylation (Figure 9, step 3) and inhibition of internalization of CSF-1–CSF-1R complexes (Figure 9, step 5) (Baccarini *et al.*, 1991; Li and Stanley, 1991). We here demonstrate that Cbl is required for both CSF-1R multiubiquitination and the second wave of CSF-1R tyrosine phosphorylation (Figure 9, step 4). Consistent with these results, both past and present, we have also shown that (i) Cbl^{-/-} cells endocytose CSF-1–CSF-1R complexes 2–3 times more slowly than Cbl^{+/+} cells and (ii) Cbl^{-/-} cells exhibit an increased efficiency of proliferation signaling that is associated with prolonged expression of the tyrosine-phosphorylated and activated CSF-1R at the cell surface.

Fig. 7. Decrease in CSF-1-induced CSF-1R multiubiquitination in Cbl^{-/-} BMM and failure of its normalization by IAA or pervanadate pre-incubation. (A) BMM were deprived of CSF-1 for 16 h and stimulated with 120 ng/ml CSF-1 for the indicated times at 37°C. CSF-1R immunoprecipitates from NP-40 cell lysates were analyzed by SDS-PAGE and Western blotting with anti-ubiquitin (anti-Ub) and anti-CSF-1R antibodies. (B) BMM were deprived of CSF-1 for 16 h and cooled to 4°C, then 8 mM IAA or pervanadate (Per-Van, 0.5 mM H₂O₂ and 0.1 mM Na orthovanadate, final concentrations) were added to the cells 10 min before stimulation with CSF-1 for 2 h at 4°C. Cells were lysed with NP-40 buffer and anti-CSF-1R antibody immunoprecipitates of the lysates subjected to gradient (8–15%) SDS-PAGE and Western blotted with the indicated antibodies. Exposure times for sections of the upper panel varied as indicated.

Effects of Cbl on macrophage morphology and proliferation

In accordance with experiments where antisense inhibition of Cbl expression or reduced Cbl phosphorylation in hck^{-/-}, fgr^{-/-}, lck^{-/-} mutant macrophages was associated with reduced macrophage spreading and migration (Meng and Lowell, 1998), CSF-1-starved Cbl^{-/-} cells were more rounded than wild-type cells and Cbl^{-/-} colonies were less dispersed and more transformed in appearance. Collectively, these results support the role of Cbl in positively regulating cellular adhesion and morphology (Meng and Lowell, 1998; Ribon *et al.*, 1998; Zell *et al.*, 1998) and in negatively regulating cellular transformation of v-abl-transformed NIH 3T3 cells (Feshchenko *et al.*, 1998).

The loss of Cbl allowed Cbl^{-/-} BMM to utilize less CSF-1, to proliferate more rapidly at limiting CSF-1 concentrations and to achieve higher cell densities in crowded cultures. In log phase growth, when growth factor is not limiting, Cbl^{-/-} and Cbl^{+/+} macrophages proliferated

Fig. 8. CSF-1R degradation in CSF-1-stimulated Cbl^{-/-} and Cbl^{+/+} BMM at 37°C. (A) Effect of methylamine and lactacystin treatment. BMM deprived of CSF-1 for 16 h were either untreated (lanes 1–4) or pre-incubated with 10 mM methylamine (lanes 5–8) or 10 μM lactacystin (lanes 9–12), prior to CSF-1 stimulation for the indicated times. CSF-1R immunoprecipitation from NP-40 lysates, SDS-PAGE and Western blotting were carried out as described in Materials and methods. (B) Inhibitory effect of lactacystin on the degradation of ubiquitinated proteins in BMM at 37°C. NP-40 cell lysates of the untreated or lactacystin-treated BMM from the experiment in (A) were resolved in SDS-PAGE and Western blotted with anti-ubiquitin antibody. (C) Graph of the data obtained by densitometric scanning of the data in (A). Bracketed regions in (A) were scanned with a computing densitometer (Molecular Dynamics) and the scanned images analyzed by MD ImageQuant Software, Version 3.3. Each point represents the percentage density of the band at that time relative to the zero time. ●, untreated Cbl^{+/+}; ○, untreated Cbl^{-/-}; ■, methylamine-treated Cbl^{+/+}; ▲, lactacystin-treated Cbl^{+/+}.

at equal rates. This is apparently due to a higher turnover of activated CSF-1Rs in Cbl^{+/+} cells, which compensates for their decreased average cell surface residence time compared with CSF-1Rs in Cbl^{-/-} cells. In contrast, the negative regulation of proliferation by Cbl in stationary phase, despite the continued increased utilization of CSF-1 by Cbl^{+/+} cells, implies that in density-arrested cells, Cbl negatively regulates proliferation in some other fashion, for example by altering the adherence requirement for proliferation (Meng and Lowell, 1998) and/or by sup-

Fig. 9. Schematic of the early CSF-1-induced changes in Cbl and the CSF-1R in macrophages at 37°C. CSF-1 binding initially leads to the rapid non-covalent dimerization and tyrosine phosphorylation of the CSF-1R (step 1), followed by the tyrosine phosphorylation of Cbl, Shc, the regulatory subunit of PI-3K (p85) and their coincident association with the CSF-1R and Grb2 in a complex (Baccarini *et al.*, 1991; Li and Stanley, 1991; Li *et al.*, 1991; Kanagasundaram *et al.*, 1996; Wang *et al.*, 1996, 1999; Husson *et al.*, 1997) (step 2). This complex is independent of the more transient CSF-1R–Sos–Grb2 complexes formed simultaneously (Wang *et al.*, 1999), although in the figure both are shown associated with one CSF-1R homodimer. The period of active non-receptor protein tyrosine phosphorylation (step 3, simultaneous with step 2) is followed by the extracellular disulfide linkage of monomers within the CSF-1R dimer that apparently permits a second wave of CSF-1R tyrosine phosphorylation, increased CSF-1R serine phosphorylation, coordinate multiubiquitination of the CSF-1R and membrane-associated Cbl (step 4), and internalization of the CSF-1–CSF-1R complex (Baccarini *et al.*, 1991; Li and Stanley, 1991; Wang *et al.*, 1996, 1999) (step 5). Tyrosine phosphorylation and multiubiquitination of the cell surface CSF-1Rs are stoichiometric (Li *et al.*, 1991; Li and Stanley, 1991; Wang *et al.*, 1999). Once internalized (step 6), both CSF-1, as shown previously (Guilbert and Stanley, 1986), and the CSF-1R are degraded intralysosomally (Figure 8) and it is assumed that the endocytosed receptor–ligand complex enters a multivesicular body (MVB) prior to entry into the lysosomal system. In contrast, ubiquitinated Cbl is not degraded but recovered in deubiquitinated form in the cytosol 3–10 min after stimulation (Wang *et al.*, 1996, 1999). Ub, ubiquitin; X, proteins tyrosine phosphorylated in the CSF-1 response; Yp, phosphotyrosine; Sp, phosphoserine.

pressing transformed cell-like growth (Feshchenko *et al.*, 1998). Thus, while Cbl-enhanced CSF-1R endocytosis explains the reduced efficiency of proliferation signaling, it appears that a separate effect of Cbl is responsible for the decreased final cell density and less transformed appearance of Cbl^{+/+} cells. The combination of these effects is likely to contribute significantly to the splenomegaly and increased frequency of splenic CSF-1-dependent macrophage colony-forming cells in Cbl^{−/−} mice (Murphy *et al.*, 1998).

Regulation of CSF-1R phosphorylation by Cbl

A large proportion of CSF-1-induced non-CSF-1R cellular tyrosine phosphorylation is not necessary for proliferation. This finding is not surprising, considering the negative regulatory role of Cbl on cell proliferation and that the decreased cellular protein tyrosine phosphorylation seen in Cbl^{−/−} cells is due primarily to the absence of Cbl itself and of proteins such as p58Shc and pp95, which have been shown to associate with Cbl in the CSF-1 response (Kanagasundaram *et al.*, 1996; Wang *et al.*, 1996, 1999). Phosphorylation of the CSF-1R was also altered in Cbl^{−/−} cells. Phosphopeptide maps of the CSF-1R from Cbl^{+/+} cells demonstrated that new sites are phosphorylated during the second wave of tyrosine phosphorylation. In the absence of Cbl, the first wave of CSF-1R tyrosine phosphorylation is qualitatively altered and reduced in intensity, and the second wave, previously associated with preparation of the CSF-1R for internalization and degradation (Baccarini *et al.*, 1991; Li and Stanley, 1991), fails to develop. It is possible that Cbl, which is known to associate with the Src-like kinases fgr and lyn in

macrophages (Meng and Lowell, 1998), transports a kinase to the CSF-1R that is responsible for the second wave of CSF-1R tyrosine phosphorylation. Alternatively, the association of Cbl with the activated CSF-1R may block the action of a phosphatase or simply enhance CSF-1R autophosphorylation.

Cbl-targeted CSF-1R multiubiquitination

Multiubiquitination of mammalian receptor tyrosine kinases is an integral part of their ligand-induced responses (Galcheva-Gargova *et al.*, 1995; Mori *et al.*, 1995a,b; Jeffers *et al.*, 1997) and is required for ligand-induced endocytosis of several membrane proteins, including yeast mating type receptors and the human growth hormone receptor (GHR) (Galan *et al.*, 1996; Hicke and Reizman, 1996; Roth and Davis, 1996; Strous *et al.*, 1996). In contrast to the ligand-induced proteasomal degradation of other tyrosine kinase receptors (Mori *et al.*, 1995b; Jeffers *et al.*, 1997; Levkowitz *et al.*, 1998), we have shown that the CSF-1R is degraded lysosomally. Thus, it is possible that Cbl-targeted CSF-1R multiubiquitination causes the increased CSF-1–CSF-1R endocytosis, as has been shown for a truncated form of the *Saccharomyces cerevisiae* α -factor receptor, Ste2p (Hicke and Reizman, 1996). As we have shown for the CSF-1R (Wang *et al.*, 1996, 1999; Figures 6B and 9), Ste2p is ubiquitinated prior to endocytosis and is degraded intralysosomally (reviewed in Hicke, 1997). However, in studies carried out with GHR-overexpressing Chinese hamster ovary (CHO) cells, conjugation of ubiquitin to the GHR is dispensable for internalization and it is the recruitment of the ubiquitin conjugation system to the GHR that is

required. Two of three models proposed to explain this result implicate the existence of a GHR-recruited adaptor protein that is multiubiquitinated coordinately with the GHR (Govers *et al.*, 1999). Since Cbl is recruited to the CSF-1R and multiubiquitinated coordinately (Wang *et al.*, 1999), it is not clear whether the multiubiquitination of Cbl, the CSF-1R or both is required for internalization, if, as seems likely, multiubiquitination is involved in CSF-1R endocytosis. As far as Cbl targeting of CSF-1R multiubiquitination is concerned, our experiments with protein tyrosine phosphatase inhibitors (Figure 7B) indicate that it is not simply the reduced level of CSF-1R tyrosine phosphorylation that prevents CSF-1R multiubiquitination in Cbl^{-/-} cells. Interestingly, Cbl possesses a UBA domain at its C-terminus (amino acids 844–884) (Hofmann and Bucher, 1996) and UBA domains have been found in a number of unrelated proteins that are either involved in the ubiquitin pathway or possess regions with homology to ubiquitin itself. Since most ubiquitin-conjugating (E2) and ubiquitin-ligating (E3) enzymes do not contain these domains, it has been suggested that proteins containing UBA domains confer target specificity for ubiquitination (Hofmann and Bucher, 1996).

Decreased endocytosis and increased signaling in Cbl^{-/-} cells

We have shown that there is prolonged cell surface expression of the activated CSF-1R in CSF-1-stimulated Cbl^{-/-} cells. Defective EGF-induced EGFR endocytosis in cells conditionally expressing a dominant-negative inhibitor of clathrin-coated vesicle formation leads to enhanced EGF-dependent proliferation (Vieira *et al.*, 1996), indicating the importance of the endocytic rate in determining the intensity of proliferation signaling. Furthermore, it has been shown that the mitogenic response of a mitogenically defective human CSF-1R (Y809F) in NIH 3T3 cells is restored by co-expressing a truncated mouse phosphatidylinositol 4-phosphate 5-kinase that stabilizes the expression of the activated mutant CSF-1R (Y809F) at the cell surface (Davis *et al.*, 1997). Thus, it is most likely that the decreased endocytosis of the CSF-1R in Cbl^{-/-} cells leads to their increased efficiency of proliferation signaling. Consistent with previous studies which indicate that the Ras/MAPK pathway is not involved in signaling macrophage proliferation (Büscher *et al.*, 1993) and that activated Cbl does not signal via Ras (Bowtell and Langdon, 1995; Ota and Samelson, 1997; Ueno *et al.*, 1997), Cbl^{-/-} and Cbl^{+/+} macrophages did not differ in their activation of Ras. Since Sos–Grb2 association with and dissociation from the CSF-1R take place before Cbl/CSF-1R multiubiquitination (Wang *et al.*, 1999) (Figure 9), the Cbl independence of Ras pathway signaling may be explained by its shut-off being early and independent of endocytosis. The prolonged signaling by Cbl^{-/-} cells involves the tyrosine phosphorylation of proteins of *M_r*s 116, 105 and 57 kDa, which have not yet been identified, and the signaling pathways regulating CSF-1-stimulated macrophage proliferation are likely to be complex (Xu *et al.*, 1993).

Relevance of Cbl enhancement of CSF-1R multiubiquitination and endocytosis

Genetic interactions between the *C.elegans* Cbl homolog, SLI-1, and another negative regulator of LET-23,

UNC-101, support our finding that Cbl is involved in receptor endocytosis. UNC-101 is the homolog of the mammalian medium chain of clathrin-associated protein, AP47. While null mutations of *sli-1* or *unc-101* alone have no overt phenotype, *sli-1 unc-101* double mutants possess a multivulva phenotype, consistent with their functional interaction (Lee *et al.*, 1994). In relevant studies in mammalian systems, overexpression of Cbl recently has been shown to enhance ligand-induced platelet-derived growth factor receptor α (PDGFR α) multiubiquitination and degradation in NIH 3T3 cells (Miyake *et al.*, 1998) and to stimulate targeting of endosomal EGFRs for degradation (Levkowitz *et al.*, 1998). However, neither of these studies addressed the question of whether Cbl regulates receptor endocytosis. The latter study, which implicated a role for Cbl in endosomal sorting, utilized CHO cells overexpressing the EGFR, in which the clathrin-dependent, rapid endocytic pathway was saturated and in which very low receptor down-regulation was observed. Thus, our results with primary cells represent the first demonstration that Cbl actually targets receptor tyrosine kinase endocytosis. They demonstrate that Cbl is required for CSF-1R multiubiquitination and confirm that this occurs at the cell surface prior to receptor endocytosis (Wang *et al.*, 1996, 1999). Most importantly, they implicate Cbl modulation of receptor tyrosine kinase endocytosis as a mechanism for the attenuation of growth factor-induced cell proliferation. The precise biochemical role of Cbl in this process remains to be elucidated.

Materials and methods

Antibodies and reagents

Goat anti-CSF-1R antiserum (Li and Stanley, 1991) to the purified mouse CSF-1R (Yeung *et al.*, 1987) was used for the cell surface CSF-1R immunoprecipitation experiments. For immunoprecipitation from cell lysates, either anti-CSF-1R antibodies affinity purified from this antiserum on a column of CSF-1R cross-linked to immobilized anti-CSF-1R peptide antibodies (Wang *et al.*, 1999), or a 1:1 mixture of two affinity-purified goat anti-mouse CSF-1R cytoplasmic domain peptide antibodies (Wang *et al.*, 1999), were used. The affinity-purified anti-mouse CSF-1R cytoplasmic domain peptide antibodies were used for Western blotting. Rabbit anti-Cbl antibody was raised against a peptide with the sequence of the mouse Cbl C-terminal 15 amino acids (LREFVSISSPAHVAT) synthesized on a multimeric antigenic peptide system resin (Wang *et al.*, 1999). Anti-PY recombinant antibody (RC20) coupled to horseradish peroxidase (for Western blotting) and monoclonal antibody PY20 (for immunoprecipitation) were obtained from Transduction Laboratories. Rabbit anti-ubiquitin antiserum was from Sigma and anti-*v-ras* (Ab-1)–agarose from Calbiochem. An Oncor Apotag peroxidase kit (Appligene Oncor Lifescreen) was used for the detection of apoptotic cells. Unless otherwise stated, all other reagents were from Sigma.

BMM preparation, culture and morphology

Cbl mutant (–/–), wild-type (+/+) and heterozygous (+/–) mice on a mixed C57Bl/6 $\times$ 129/SvJ background were maintained and bred in the barrier facility at Albert Einstein College of Medicine as described previously (Murphy *et al.*, 1998). Genotype analysis was performed by Southern blot analysis of *Bam*HI-digested tail DNA from pups using an *Xba*I–*Sal*I genomic fragment from outside the targeting vector as the probe (Murphy *et al.*, 1998). Day 5 BMM from Cbl^{+/+} and Cbl^{-/-} mice were prepared and cultured in medium containing 15% fetal calf serum (Bio-Whittaker) and 120 ng/ml human recombinant CSF-1 (a gift from Chiron Corp.) as described previously (Tushinski *et al.*, 1982; Stanley, 1990). For studies of colony morphology, cells were seeded at 500 cells per 35 mm culture dish (Becton Dickinson) and cultured for 8 days. Colonies were photographed under phase-contrast and the cells fixed with 0.2% methylene blue in methanol for additional photomicrography. For scanning electron microscopy (SEM), cells were grown on coverslips,

fixed for 5 s in 1% osmium tetroxide and 2×60 min in 2.5% glutaraldehyde and processed for SEM. To compare growth rates in non-limiting CSF-1 and survival without CSF-1, day 5 BMM were washed with α -minimal essential medium (α -MEM) and cultured with or without 120 ng/ml CSF-1. Cells were fed every 2 days for 8 days. Each day, cells from triplicate plates were counted using a Coulter Counter. To compare growth rates at limiting CSF-1 concentrations and to determine the CSF-1 utilization rates, day 3 BMM (Tushinski *et al.*, 1982; Stanley, 1990) were seeded at 4×10^4 cells per 35 mm plate. On day 0, the cells were washed with α -MEM and then cultured with or without CSF-1 for 6 days and the cell culture medium replaced with fresh medium daily. On days 0, 5 and 6, cells from triplicate plates were counted. On day 6, the media were collected from the plates cultured for 24 h after the day 5 medium change and assayed in duplicate for CSF-1 by radioimmunoassay (Stanley, 1979), together with samples of fresh medium, in order to assess the CSF-1 utilization rate.

Ras GTP loading experiments

Subconfluent BMM (3×10^6 /60 mm dish), deprived of CSF-1 for 16 h (upregulated), were labeled with [^{32} P]phosphate as described previously (Li *et al.*, 1991). Cells were incubated at 37°C with different amounts of CSF-1 for 2 min or with 120 ng/ml CSF-1 for various times. Cell lysis, recovery of Ras by binding to anti-*v-ras* (Ab-1)-agarose, elution and separation of the eluate by thin-layer chromatography were as described (Xu *et al.*, 1993). Radioactivity in spots corresponding to GDP and GTP was quantitated using a PhosphorImager (Molecular Dynamics).

CSF-1 stimulation and analysis of stimulated BMM

Upregulation and stimulation of subconfluent 100 mm dish cultures of BMM with 360 ng/ml purified human recombinant CSF-1 at 37 or 4°C and their solubilization in lysis buffer (1% NP-40, 10 mM Tris-HCl, 50 mM NaCl, 30 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, 100 μM Na_3VO_4 , 5 μM ZnCl_2 , 1 mM benzamide, 10 $\mu\text{g/ml}$ leupeptin and 10 $\mu\text{g/ml}$ aprotinin, pH 7.2) or SDS sample buffer (Laemmli, 1970) were performed as described previously (Li and Stanley, 1991; Li *et al.*, 1991). For immunoprecipitations, lysates were incubated with anti-PY or anti-CSF-1R antibodies for 1 h at 4°C and the antibodies collected following a subsequent 1 h incubation with protein G-Sepharose (Zymed) at 4°C. Protein determinations, gradient (6–16% acrylamide, except where otherwise specified) SDS-PAGE (Laemmli, 1970) and Western blotting were carried out as described previously (Yeung *et al.*, 1992; Wang *et al.*, 1996). For the proteasomal and lysosomal inhibitor experiments, BMM were prepared for CSF-1 stimulation and solubilization as described above, with the exception that they were incubated with either 10 μM lactacystin, 40 μM MG132 (Calbiochem), 100 μM chloroquine, 1 mM leupeptin (Sigma) or 10 mM methylamine (Sigma) at 37°C for 1 h before and during CSF-1 treatment. Anti-CSF-1R immunoprecipitates were processed as described above. The between-experiment variation in the levels of the total CSF-1R following stimulation were due to variations in the method used (direct Western blot of lysates or immunoprecipitation) and also in the cell populations, culture conditions and cell densities. All comparisons between *Cbl*^{-/-} and *Cbl*^{+/+} cells were made within experiments.

[^{125} I]CSF-1 binding and uptake assays

Mouse CSF-1 was purified, radioiodinated (340 000 c.p.m./ng) and the saturation binding carried out with different concentrations of [^{125} I]CSF-1 at 4°C for 7 h, as described previously (Stanley, 1985). As the binding of CSF-1 to the CSF-1R is irreversible at 4°C, it is not possible to use equilibrium methods to determine dissociation constants (Stanley and Guilbert, 1981). On-rate and off-rate constants were determined as described previously (Guilbert and Stanley, 1986). For [^{125} I]CSF-1 uptake at 37°C, cells were incubated with 120 ng/ml of mouse CSF-1 containing 5×10^5 c.p.m./ml [^{125} I]CSF-1 in the presence and absence of a 500-fold excess of unlabeled human CSF-1 for various times up to 10 min. The cells were washed four times in ice-cold phosphate-buffered saline (PBS) and cell surface-bound [^{125} I]CSF-1 was removed by incubating cells in ice-cold pH 4.0 dissociation buffer (0.1% newborn calf serum in 100 mM acetic acid, 10 mM NaCl, pH 4.0) for 20 min. Pelleted cells were lysed in 0.1 M NaOH and counted for [^{125} I] in a gamma counter. Specific uptake data, in triplicate, were normalized for protein content of the lysate (Guilbert and Stanley, 1986).

Cell surface, internal and total CSF-1R immunoprecipitation

Upregulated BMM were incubated in the presence or absence of CSF-1 at 37°C for various times. To isolate cell surface and internal CSF-1Rs (Li and Stanley, 1991), cells were washed three times with ice-cold PBS

and incubated with 3.0 ml of PBS containing 10% goat anti-CSF-1R antiserum for 15 min at 4°C to occupy all cell surface CSF-1Rs. The antiserum was removed and the cells washed five times with 4 ml of ice-cold PBS prior to solubilization in lysis buffer and centrifugation of the lysate at 13 000 g for 30 min at 4°C. The protein contents of the supernatants were equalized and the cell surface CSF-1R antibody complexes were isolated by incubation with recombinant protein G-Sepharose at 4°C for 1 h, prior to collection by centrifugation (13 000 g, 5 min, 4°C). The resulting supernatants were incubated with anti-CSF-1R antibody for 1 h at 4°C, then with recombinant protein G-Sepharose for 1 h at 4°C to immunoprecipitate the internal pool of CSF-1R. The Sepharose bead pellets containing the cell surface or the internal pool of CSF-1Rs were washed five times in lysis buffer, followed by two washes in the same buffer without NP-40. Total CSF-1R immunoprecipitations were performed on a parallel set of CSF-1-stimulated cells, solubilized in lysis buffer but not subjected to the surface antibody-binding step. In all cases, the immunoprecipitated CSF-1R was eluted with SDS sample buffer at 65°C for 10 min.

Phosphopeptide mapping

The ^{32} P-labeling, CSF-1 stimulation and anti-CSF-1R immunoprecipitation were carried out as described previously (Li *et al.*, 1991). The CSF-1R proteins were eluted from the beads with 2% SDS in 50 mM Tris-HCl, pH 6.8, reduced (100°C, 3 min) in 10 mM dithiothreitol (DTT) and alkylated (20°C, 20 min) with 20 mM IAA prior to separation by gradient (5–15% acrylamide, polymerized on GelBond PAG film; FMC BioProducts) SDS-PAGE and wet gel autoradiography to locate the labeled CSF-1R bands. The gel segment containing the labeled receptor was excised, washed thoroughly with 50 mM NH_4HCO_3 , pH 8.0, cut into small pieces and digested with trypsin (Promega) at 37°C for 18 h in 50 mM NH_4HCO_3 , pH 8.0 containing 5% acetonitrile and 1 mM CaCl_2 . The whole digestion mixture was dried in a SpeedVac (Savant). The tryptic peptides were extracted with 70% formic acid at room temperature, dried in a SpeedVac and dissolved in 5 μl of the pH 1.9 buffer (Van der Geer and Hunter, 1990). The dissolved peptides were separated in two dimensions on a cellulose thin-layer plate (Eastman Kodak Company) using the Hunter Thin Layer Electrophoresis System (Model HTLE-7000, C.B.S. Scientific Company, Inc.), as described previously (Van der Geer and Hunter, 1990).

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