c-Cbl Is Transiently Tyrosine-phosphorylated, Ubiquitinated, and Membrane-targeted following CSF-1 Stimulation of Macrophages*

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Early colony stimulating factor-1 (CSF-1)-induced changes in the behavior of p120c-cbl in mouse BAC1.2F5 macrophages were investigated. p120c-cbl is associated with Grb2 in the cytoplasm of unstimulated cells. Following a 1 min stimulation with CSF-1, p120c-cbl becomes tyrosine-phosphorylated and associates with tyrosine-phosphorylated Shc and an unknown phosphotyrosyl protein (pp80). Simultaneously, it is ubiquitinated and translocated to the membrane. By 10 min of stimulation, this c-Cbl exhibits substantially decreased tyrosine phosphorylation and is de-ubiquitinated and relocated in the cytosol. However, the association of p120c-cbl with Shc persists for at least 60 min. These data indicate that signaling via the CSF-1R involves the transient modification of p120c-cbl and its recruitment as a complex to membrane.

Colon-stimulating factor-1 (CSF-1) regulates the survival, proliferation, and differentiation of mononuclear phagocytic cells via its tyrosine kinase receptor, the c-fms protooncogene product (reviewed in Ref. 1). Incubation of myeloid cells with CSF-1 activates the CSF-1 receptor (CSF-1R) kinase leading to its rapid autophosphorylation and the subsequent tyrosine phosphorylation of certain cellular proteins (2), including protein-tyrosine phosphatase 1C (3) and Shc (4).

The c-Cbl protooncogene product, a 120-kDa protein, was originally identified as the cellular homologue of the transforming protein of the murine CaS NS-1 retrovirus that induces pro-B, pre-B, and myeloid tumors in mice. It is primarily expressed in hematopoietic cells (5, 6). Analysis of the sequence reveals that Cbl has a potential nuclear localization sequence, a region of high negative charge, a potential RING finger domain, a proline-rich region, and a leucine zipper-like motif (7). Several recent studies have shown that c-Cbl is tyrosine-phosphorylated in T cell receptor signaling (8, 9) in cells expressing the v-abl and bcr-abl oncogenes (10), Fc receptor engagement (11), and granulocyte-macrophage colony-stimulating factor and erythropoietin signaling (12). p120c-cbl is found in macrophages (13, 14) and has recently been shown to be tyrosine-phosphorylated in response to CSF-1 (14). In this communication, we show that CSF-1 stimulation of macrophages results in rapid, transient, tyrosine phosphorylation, membrane translocation, and ubiquitination of p120c-cbl and the formation of a longer lived p120c-cbl complex with Shc.

MATERIALS AND METHODS

Cell Culture and Fractionation—Up-regulation and stimulation of BAC1.2F5 macrophages (grown in 100-mm tissue culture dishes) with CSF-1 (recombinant human CSF-1, a gift from Chiron Corp.) at 37 °C and their solubilization with Nonidet P-40 or SDS were performed as described previously (15, 16). For subcellular fractionation, cells were washed once with cold phosphate-buffered saline (136 mM NaCl, 3 mM KCl, 8 mM NaH2PO4, 1.5 mM KH2PO4, pH 7.4), collected by scraping in phosphate-buffered saline containing 0.1 mM sodium orthovanadate and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and pelleted by centrifugation at 400 × g at 4 °C. The cell pellet was suspended in 8 volumes of hypotonic buffer (55 mM Tris-HCl, pH 7.5, 10 mM sodium pyrophosphate, 10 mM NaF, 0.5 mM sodium orthovanadate, 1 mM sodium iodoacetate, 0.1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Subsequent homogenization and subcellular fractionation were performed as described (17), except that the compensation buffer was composed of 20 mM Tris-HCl, pH 7.0, 0.95 mM sucrose, 30 mM sodium pyrophosphate, 100 mM NaF, 100 mM NaCl, 0.5 mM sodium orthovanadate, 0.025 mM ZnCl2, 1% (v/v) Methylene blue labeling was performed as described earlier (15), and the effect of cycloheximide on the protein synthetic rate was assessed by liquid scintillation counting of trichloroacetic acid precipitates.

Antibodies, Immunoprecipitation, and Immunoblotting—The rabbit anti-Cbl polyclonal antisera (R2) was raised against a glutathione S-transferase-human Cbl fusion protein purified and affinity purified by affinity chromatography as described previously (18). Anti-phosphotyrosine (anti-Tyr(P)) antibody RC20 and polyclonal anti-Shc and anti-Grb2 antibodies were purchased from Transduction Laboratories and the monoclonal anti-Grb2 antibody from Upstate Biotechnology Inc. The anti-ubiquitin antisera was purchased from Sigma. Affinity-purified rabbit polyclonal antibody against SDS-denatured ubiquitin was a gift from Dr. A. L. Haas. Anti-Cbl antibody (C15), directed to the carboxy-terminal 15 amino acids of p120c-cbl was purchased from Santa Cruz Biotechnology, Inc. Immunoprecipitation and immunoblotting were performed as described previously (3). Immunoprecipitations for different time points were carried out from equivalent amounts of protein. ECL reagent (Amer sham Corp.) was used for the detection of the immunoreactive bands on blots. For reprobing, anti-Tyr(P) blots were stripped with 1 μM NaCl containing 10 mM Tris-HCl, pH 7.0, and the other immunoblots with 2% SDS containing 0.1 μM β-mercaptoethanol and 50 mM Tris-HCl, pH 7.0.

Dephosphorylation—Phosphatase inhibitors (pyrophosphate, fluoride, iodoacetate, zinc chloride, and vanadate) were removed from samples prior to their dephosphorylation by dialysis (or washing in the case of immunoprecipitates and membrane fractions) in 10 mM Tris-HCl, 0.14 mM NaCl, 0.1 mM PMSF, pH 7.4. Samples (400 μg) were then adjusted to 50 mM Tris-HCl, 5 mM MgCl2, and 0.1 mM PMSF, pH 9.0. Alkaline phosphatase (20 units, Sigma) was added to each sample, and dephosphorylation was carried out for 50 min at 37 °C. The reaction was stopped by the addition of 5 × SDS sample buffer.

Other Techniques—Proteins were resolved by gradient SDS-poly-
CSF-1-induced Modification and Translocation of p120c-cbl

RESULTS AND DISCUSSION

p120c-cbl Is Tyrosine-phosphorylated in Response to CSF-1 Stimulation—Upon CSF-1 stimulation of BAC1.2F5 macrophages, a number of proteins are highly tyrosine-phosphorylated within 1 min at 37 °C (Fig. 1A, lanes 1 and 2). One of these, a ~120-kDa tyrosine-phosphorylated protein, was shown to co-migrate with p120c-cbl in SDS-polyacrylamide gel electrophoresis (Fig. 1, A and B, lanes 1 and 2). Anti-phosphotyrosine Western blots of lysates, which had been dephosphorylated by anti-Cbl immunoprecipitation revealed that p120c-cbl was the major tyrosine-phosphorylated band of 120 kDa in CSF-1-stimulated cells (data not shown). Anti-phosphotyrosine Western blots of anti-Cbl immunoprecipitates from cell lysates prepared at various times after CSF-1 stimulation showed that p120c-cbl is rapidly and transiently (peak, 1–3 min) tyrosine-phosphorylated (Fig. 1A, lanes 5–10). However, when the same blot was reprobed with anti-Cbl antibody, decreased p120c-cbl protein was detected at the 1 (56% of zero time) and 3 (39% of zero time) min time points (Fig. 1B, lanes 6 and 7), when p120c-cbl was most highly tyrosine-phosphorylated. Similar results were obtained following Fcγ receptor clustering in macrophages (14). Reasons for this apparent decrease in p120c-cbl protein are given below.

p120c-cbl Associates with Shc and Grb2—p120c-cbl has been shown to associate in vitro with the SH2 domains of the Fyn, Lck, and Blk tyrosine kinases (8) and in vivo with the Lyn and Syk tyrosine kinases in HL60 cells (11) and other proteins, including GTPase-activating protein, phospholipase Cγ (8), Crk (9), and Grb2 (8, 12) via their SH2 or SH3 domains in vitro and/or in vivo. p120c-cbl could not be precipitated from activated T cell lysates by a glutathione S-transferase-Shc SH2 fusion protein (9). However, as shown in Fig. 1A, lanes 5–10, a 56-kDa tyrosine-phosphorylated protein was co-precipitated with p120c-cbl from CSF-1-stimulated BAC1.2F5 cell lysates, and reprobing of the same blot with anti-Shc antibody (Fig. 1C, lanes 5–10) confirmed that CSF-1 treatment stimulated association of p56 Shc with p120c-cbl. This association, possibly via the Shc phosphotyrosine interaction domain (reviewed in Ref. 19), was maintained for up to 60 min.

p120c-cbl was previously shown to associate with Grb2 in J urkat cells (8) and cells of the human leukemia cell line UT-7 (12) via the Grb2 SH3 domains. In BAC1.2F5 macrophages, Grb2 co-precipitates with p120c-cbl, independently of CSF-1 stimulation (Fig. 1D, lanes 5–10). A 120-kDa tyrosine-phosphorylated protein co-precipitated with both Shc and Grb2 after CSF-1 stimulation when they were separately immunoprecipitated from cell lysates (Fig. 1A, lanes 11–14). However, when these blots were reprobed with anti-Cbl antibody, p120c-cbl was not detected, possibly because of insufficient amounts and/or reduced detection of p120c-cbl from stimulated cells (see below).

In an additional experiment at 37 °C, p120c-cbl also exhibited CSF-1-dependent association with a tyrosine-phosphorylated protein of 80 kDa (pp80) (data not shown). All of these associations were more obvious following CSF-1 stimulation at 4 °C, where all three co-precipitated proteins were more concentrated in the membrane fraction than in cytosol (see below, Fig. 3).

Translocation of the p120c-cbl to Membrane—To determine whether there was any change in the subcellular distribution of p120c-cbl on stimulation with CSF-1 at 37 °C, cells were separated into membrane and cytosolic fractions at various times after stimulation and the fractions Western blotted with anti-Cbl (Fig. 2A, lanes 1–4 and 9–12). Within 1 min of stimulation there was an apparent reduction of p120c-cbl in the cytosolic fraction. While a very small amount of p120c-cbl was detected in the membrane fraction at this time, it did not compensate for the apparent loss (up to 80%) from the cytosol. Because earlier experiments had suggested that the tyrosine-phosphorylated form of p120c-cbl might not be detected as efficiently by anti-Cbl antibodies as the non-tyrosine-phosphorylated form (Fig. 1A, lanes 6 and 7), cytosolic and membrane fractions from the time course (Fig. 2A, lanes 1–4 and 9–12) were treated with alkaline phosphatase to dephosphorylate the protein (Fig. 2A, lanes 5–8 and 13–16) prior to the anti-Cbl Western blot analysis. While dephosphorylation improved the detection by anti-Cbl (Fig. 2A, lanes 5–8 and 13–16) and clearly demonstrated translocation of p120c-cbl to the membrane fraction, the amount of p120c-cbl detected in the membrane fraction at 1 min was still not equivalent to the amount lost from the cytosolic fraction at this time (note longer exposure times for the membrane blots in Fig. 2A).

In addition, the effect of alkaline phosphatase treatment was not selective for tyrosine-phosphorylated p120c-cbl from stimulated cells, since improvement in detection was also seen following treatment of lysates from unstimulated cells (Fig. 2A, lanes 1 and 5).

To obtain information concerning the mechanism of the apparent CSF-1-induced loss of p120c-cbl and to further study the
CSF-1-induced Modification and Translocation of p120<sup>c-cbl</sup>

Fractions of p120<sup>c-cbl</sup>, cells were stimulated for different times at 4°C, which slows the rates of early changes, maximizes the extent and yield of tyrosine-phosphorylated proteins, and blocks internalization of the ligand-receptor complex (reviewed in Ref. 1). Whole cell lysates and cytosolic and membrane fractions were Western blotted with anti-Cbl (Fig. 2B). CSF-1 stimulation at 4°C resulted in the generation of additional p130<sup>c-cbl</sup>-p140<sup>c-cbl</sup> species of Cbl (Fig. 2B, lanes 6, 8, and 10) over a time period (120 min) previously shown to be equivalent to ~30 s of stimulation at 37°C (2, 15). When cells stimulated at 4°C for 120 min were brought back to 37°C, the higher M<sub>r</sub> species rapidly disappeared in 3 min, and the amount of p120<sup>c-cbl</sup> approximated unstimulated levels by 10 min (Fig. 2C). The higher M<sub>r</sub> forms were present in both the cytosolic and membrane fractions but selectively concentrated in the membrane fraction (Fig. 2B, lanes 8 and 10) and persisted even after alkaline phosphatase dephosphorylation of the samples (data not shown). As in the case of stimulation at 37°C (data not shown), no Cbl was detected in the cytoskeleton (Fig. 2B, lanes 11 and 12) or nuclear (Fig. 2B, lanes 13 and 14) fractions. In contrast to the results obtained at 37°C, where CSF-1-induced translocation to the membrane was apparently <5% (Fig. 2A, lanes 5–8 and 13–16), at 4°C there was a high degree of translocation (~50%, Fig. 2B, lanes 7–10). Thus these ~4°C data support the 37°C data, indicating that there is a rapid, CSF-1-induced translocation of c-Cbl to the membrane that is associated with the generation of forms exhibiting increased apparent M<sub>r</sub> values that are barely visible at 37°C (Fig. 2A, lane 14), possibly because of their instability or further modification at this temperature. However, cycloheximide (10 μg/ml) pretreatment of cells at 37°C failed to block the recovery of p120<sup>c-cbl</sup> after CSF-1 stimulation despite the fact that in a parallel experiment it inhibited the incorporation of <sup>[35S]</sup>methionine by 40-fold (data not shown). This observation and the results from the 4–37°C temperature shift experiment (Fig. 2C) clearly show that the transient loss of p120<sup>c-cbl</sup> is not due to protein degradation but to a reversible modification that results in dispersion of the c-Cbl protein bands and/or inhibition of its detection by antibody.

CSF-1 Stimulates Ubiquitination of p120<sup>c-cbl</sup>-Anti-tyrosine-phosphorylated Western blots of anti-Cbl immunoprecipitates from the cytosolic and membrane fractions of cells stimulated with 13.2 nM CSF-1 for 1 min at 37°C or 120 min at 4°C were fractionated into cytosolic (Cyt) and Nonidet P-40 solubilized membrane (Mem) fractions. Anti-Cbl immunoprecipitates of these fractions were Western blotted (WB) with anti-Tyr(P) (PY) (A) and reprobed with anti-Cbl (B), anti-ubiquitin (C), anti-Shc (D), and anti-Grb2 (E). The dark shadow on the left side of C (lane 1) is an artifact. Controls for the specificity of the ubiquitin staining are described in the text.
with anti-Cbl (Fig. 3B) indicated that upon stimulation at 4 °C, a significant proportion of p120-cbl had translocated to the membrane where it was recovered as higher molecular weight, tyrosine-phosphorylated forms. As the heterogeneity of the latter species might be explained by ubiquitination and the apparently decreased levels of p120-cbl observed at 1 and 3 min of stimulation at 37 °C (Figs. 1B and 2A) by dispersion due to further multiquitination, the same blot was reprobed with anti-ubiquitin antibodies (Fig. 3C). For the 4 °C experiments (Fig. 3C, lanes 5–8), an intense band, corresponding to the 130–140-kDa anti-Cbl Western blotted bands from the membrane fraction of stimulated cells (Fig. 3B, lane 8), was seen, together with smeared, continuous staining in the range of 130 kDa to >300 kDa (Fig. 3C, lane 8). Such smearing is characteristic of multiquitination. Two additional experiments were carried out in order to confirm the specificity of the ubiquitin staining. Preincubation of the anti-ubiquitin antibody with ubiquitin (0.5 mg/ml) completely blocked the staining, and identical results were obtained when blots were reprobed with affinity-purified anti-ubiquitin antibody prepared as described by Haas and Bright (20) (data not shown). In addition, similar results to those shown in Fig. 3C were obtained when another anti-Cbl antibody (C15) was used in the immunoprecipitation (data not shown). For experiments carried out at 37 °C (Fig. 3C, lanes 1–4), lighter anti-ubiquitin staining bands were seen in the cytosolic as well as the membrane fraction from stimulated cells. In the membrane fraction, these bands corresponded to the highest M₈ bands from the membrane fraction of cells stimulated at 4 °C, while the cytosolic fraction contained bands of 130–140 kDa. When samples pretreated with 1% SDS and reconstituted with Nonidet P-40 (final detergent concentration, 0.1% SDS, 1% Nonidet P-40) were used in a similar experiment, a similar pattern of ubiquitination was observed, although less c-Cbl was immunoprecipitated by the antibody possibly due to interference by tightly bound SDS (data not shown). Thus these data indicate that p120-cbl is rapidly and transiently tyrosine-phosphorylated, ubiquitinated, and translocated to the membrane upon CSF-1 stimulation and that its association with the membrane occurs during the peak of CSF-1R signaling prior to degradation of the receptor-ligand complex (21). During this process, it becomes associated with tyrosine-phosphorylated Shc (Fig. 3, A and D) and a tyrosine-phosphorylated protein, pp80 (Fig. 3A), and exhibits increased association with Grb2 (Fig. 3E). Upon its release to the cytoplasm, it continues to remain associated with tyrosine-phosphorylated Shc (Fig. 1C). The transient, apparent loss of anti-Cbl-reactive protein is not unique to stimulation by CSF-1. It has been observed during epidermal growth factor stimulation, T cell receptor activation, 2 and Fcγ receptor clustering (14), suggesting that ubiquitination of p120-cbl may be a general response.

The existence of stable multiquitinated proteins (22) and specific deubiquitinating enzymes (23) suggests that ubiquitin may have other functions besides targeting proteins for degradation. Recent studies indicate that p120-cbl is the homologue of the product of the Caenorhabditis elegans gene, sl-1 (suppressor of cell lineage defect-1) (26). sl-1 negatively regulates signaling via the epidermal growth factor receptor homologue LET-23, which regulates vulval induction. On the basis of genetic studies, it has been suggested that SLI-1 acts as a negative regulator of the vulval induction pathway at a step prior to LIN-45 RAF, most likely at the LET-23/SEM-5 step (26, 27). We have shown that p120-cbl interacts with the SEM-5 homologue Grb2 in the cytoplasm of unstimulated cells and that following stimulation with CSF-1, it is modified and translocated to the membrane. Its association with tyrosine-phosphorylated Shc is consistent with an action of p120-cbl upstream of Ras, since Shc, via its association with Grb2, mediates activation of Sos (19). In support of this, we have recently observed that the initial membrane association of p120-cbl involves the activated CSF-1R. Of relevance to a possible negative regulatory role of p120-cbl in CSF-1 signaling, we have also shown that the rapid CSF-1-induced modification increasing the M₈ of the CSF-1R (21) prior to its internalization is ubiquitination, which may be associated with CSF-1R degradation. It is possible that one of the functions of p120-cbl is the targeting of CSF-1R ubiquitination.

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