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CSF-1 Stimulated Multiubiquitination of the CSF-1 Receptor and of Cbl Follows Their Tyrosine Phosphorylation and Association With Other Signaling Proteins

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Abstract Addition of colony stimulating factor-1 (CSF-1) to macrophages stimulates the rapid, transient tyrosine phosphorylation, membrane association and multiubiquitination of Cbl (Wang et al. [1996] J. Biol. Chem. 271:17–20). Kinetic analysis reveals that the tyrosine phosphorylation of Cbl is coincident with its plasma membrane translocation and association with the activated tyrosine phosphorylated CSF-1R, p85, Grb2, and tyrosine phosphorylated p58Shc and that these events precede the simultaneous multiubiquitination of Cbl and the CSF-1R. Tyrosine phosphorylation and multiubiquitination of the cell surface CSF-1R are stoichiometric and the multiubiquitinated CSF-1R is degraded. Similarly, the membrane associated Cbl is almost stoichiometrically ubiquitinated, but the ubiquitinated Cbl is not degraded, being recovered, deubiquitinated, in the cytosol 3–10 min after stimulation at 37°C. In the membrane fraction of cells stimulated at 4°C, the association of p58Shc and Grb2 with Cbl is stable, whereas its association with Sos and p85 is transient and their dissociation occurs at the time CSF-1R and Cbl multiubiquitination commence. The membrane translocation and the pattern of association of Sos with the CSF-1R, p85, Grb2, and p58Shc resemble those of Cbl but Sos is not tyrosine phosphorylated, nor multiubiquitinated and the coprecipitation of these proteins, other than Grb2, with Sos is much less. Complexes formed by Sos and Cbl are largely independent and membrane complexes of Cbl with other tyrosine phosphorylated proteins, p85 and Grb2 also contain CSF-1R. These data raise the possibility that the predicted negative regulatory role of Cbl in macrophages is its enhancement of ligand-induced CSF-1R internalization/degradation. J. Cell. Biochem. 72:119–134, 1999.

Key words: colony stimulating factor-1; Cbl; receptor tyrosine kinase; multiubiquitination; signal transduction

Colony stimulating factor-1 (CSF-1) is the primary regulator of macrophage and osteoclast development [reviewed in Stanley, 1994; Pollard and Stanley, 1996]. Regulation of mononuclear phagocyte survival, proliferation and differentiation by CSF-1 is mediated by the CSF-1 receptor (CSF-1R) tyrosine kinase, which is encoded by the c-*fms* proto-oncogene product [Guilbert and Stanley, 1980; Byrne et al., 1981; Sherr et al., 1985; Yeung et al., 1987]. Early events in CSF-1 signal transduction have been studied in myeloid cells and fibroblasts ectopically expressing the CSF-1R as well as in macrophages [reviewed in Stanley, 1994; Roussel, 1994]. Detailed kinetic analysis of the changes in the CSF-1R and cellular protein tyrosine phosphorylation within the first minute of addition of CSF-1 were studied in CSF-1-dependent BAC1.2F5 macrophages [Sengupta et al., 1988; Li and Stanley, 1991; Li et al., 1991; Baccarini et al., 1991]. CSF-1 binding initially causes noncovalent dimerization and tyrosine phos-

Abbreviations used: CSF-1, colony stimulating factor-1; CSF-1R, colony stimulating factor-1 receptor; EGF, epidermal growth factor; PY, phosphotyrosine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PDGFR, platelet derived growth factor receptor; PI 3-kinase, phosphatidylinositide 3-kinase; p85, regulatory subunit of PI 3-kinase; PIPES, piperazine-N,N′-bis[2-ethanesulfonic acid]; FITC, Fluorescein isothiocyanate; IgG, Immunoglobulin G; BSA, bovine serum albumin; PTB, phosphotyrosine binding.

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phorylation of the CSF-1R. This is followed by the tyrosine phosphorylation of other cellular proteins, which are primarily cytoplasmic. Some of these tyrosine phosphorylated proteins and other signaling molecules, including Grb2, Shc, and the p85 regulatory subunit of phosphatidyl inositol 3′ kinase (p85) associate with the CSF-1R [Husson et al., 1997, reviewed in Hamilton, 1997]. The CSF-1R dimer then becomes covalently associated, at least transiently, via inter-receptor disulfide bonds. This is followed by a further increase in tyrosine and serine phosphorylation of the CSF-1R, a substantial increase in the molecular mass of one of the CSF-1R monomers and internalization of the CSF-1/CSF-1R complex. Inhibition of inter-receptor disulfide bond formation by treatment of cells with a carboxymethylating agent blocks these latter events, suggesting that the covalent disulfide linkage of the receptor monomers triggers them [Li and Stanley, 1991].

One of the major proteins that is tyrosine phosphorylated in response to CSF-1 is $p120^{c\text{-}cbl}$, the cellular homologue of the transforming protein encoded by the cas NS-1 retrovirus that induces pro-B, pre-B and myeloid tumors in mice. Primarily expressed in hematopoietic cells [Langdon et al., 1989], $p120^{c\text{-}cbl}$ has been shown to be tyrosine phosphorylated in responses mediated by both tyrosine kinase and non-tyrosine kinase receptors and has been shown to associate in a ligand-dependent manner with both the epidermal growth factor (EGF) and stem cell factor receptor tyrosine kinases [Galisteo et al., 1995; Meisner and Czech, 1995; Brizzi et al., 1996]. c-Cbl contains several important protein-protein interaction domains including a phosphotyrosine binding (PTB) domain, a ring finger domain, several proline-rich SH3 binding domains, and a carboxyterminal leucine zipper domain [Blake et al., 1991; Thien and Langdon, 1997]. Often associated with Grb2 in unstimulated cells, c-Cbl has been reported to associate with Grb2, Crk, Nck, Shc, p85, and 14–3-3 proteins as well as Abl, Src, Syk, Fyn, and Zap-70 tyrosine kinases in activated cells [Donovan et al., 1996; Ribon et al., 1996; De Jong et al., 1995; Buday et al., 1996; Smit et al., 1996; Soltoff and Cantley, 1996; Kim et al., 1995; Meisner et al., 1995; Liu et al., 1996; Andoniou et al., 1994, 1996; Tanaka et al., 1996; Ota et al., 1996; Tsygankov et al., 1996; Eid et al., 1995].

The homologues of Cbl in *Caenorhabditis elegans* (suppressor of lineage defect-1; SLI-1) [Yoon et al., 1995; Jongeward et al., 1995]) and Drosophila (D-Cbl) [Meisner et al., 1997] have been reported to possess negative regulatory roles in receptor tyrosine kinase signaling. Genetic studies indicate that SLI-1 acts as a negative regulator of vulval induction, most likely at the LET-23 (EGF receptor homologue)/SEM-5 (Grb2 homologue) step of this Ras pathway [Jongeward et al., 1995]. Consistent with the interaction of Cbl with activated tyrosine kinase receptors [Galisteo et al., 1995; Meisner and Czech, 1995; Brizzi et al., 1996] and of D-Cbl with the *Drosophila* EGF receptor [Meisner et al., 1997], null mutations of SLI-1 have no effect on either a *let-23* wild type or null background, but suppress the phenotypes of hypomorphic *let-23* alleles [Jongeward et al., 1995]. The function of Cbl and the mechanism of its action in mammalian cells are not clear. However, experiments are consistent with a negative regulatory role that does not necessarily inhibit Ras activation [Ota and Samelson, 1997; Ueno et al., 1997].

In a previous study, we have shown that CSF-1 stimulation of macrophages leads to the rapid, transient tyrosine phosphorylation of Cbl, its transient association with the membrane fraction, and its transient multiubiquitination without degradation [Wang et al., 1996]. While the multiubiquitination of proteins has generally been associated with their targeting for degradation via the cytoplasmic proteosomal system, several multiubiquitinated proteins are stable [Wu et al., 1981] and a large number of specific deubiquitinating enzymes have been identified [Hochstrasser, 1996]. Furthermore, there is an increasing number of reports describing proteins that are reversibly multiubiquinated without being destroyed [Hochstrasser, 1996; Paolini and Kinet, 1993; Zhaung and McCauley, 1989; Chen et al., 1996; Hicke and Reizman, 1996]. To further investigate the CSF-1 induced membrane association and multiubiquitination of Cbl, we have studied the kinetics of Cbl tyrosine phosphorylation, membrane association, and ubiquitination in relation to the early CSF-1-induced changes in the CSF-1R, the association of Cbl with the CSF-1R and the association of Cbl, and the CSF-1R with other signaling proteins. Cbl tyrosine phosphorylation, translocation to the plasma membrane, and association with the activated

CSF-1R and other signaling molecules are events that are temporally associated with the first wave of CSF-1R tyrosine phosphorylation. In contrast, the multiubiquitination of Cbl is temporally associated with changes, following the disulfide bonding of receptor monomer units within the CSF-1R dimer, that apparently lead to a second wave of tyrosine phosphorylation and simultaneous multiubiquitination of the CSF-1R.

MATERIALS AND METHODS

Reagents and Antibodies

Phosphotyrosine (PY) was from Sigma (St. Louis, MO). Peptides were synthesized in the Laboratory of Macromolecular Analysis of the Albert Einstein College of Medicine. A mSos proline-rich peptide (EVPVPPPVPPPRRRE) [Meisner and Czech, 1995] was synthesized and purified by reverse phase high pressure liquid chromatography. Rabbit anti-Cbl antibody was raised against a peptide with the sequence of the mouse Cbl carboxy-terminal 15 amino-acids (LREFVSISSPAHVAT) synthesized on a multimeric antigenic peptide system resin. Anti-CSF-1R antibodies against two cytoplasmic domain peptides (an interkinase domain peptide: EGDSSYKNIHLEKKYVRRDSGFC, and a carboxy-terminal domain peptide: NNDGDYANLPSSGGSGSDSC) were raised in a goat by immunizing with a mixture of the two Keyhole Limpet Hemocyanin coupled peptides [Büscher et al., 1993]. The antibodies specific to each peptide were purified from the serum by peptide affinity chromatography. In the case of the anti-CSF-1R antibodies, a 1:1 mixture of the two affinity purified antibodies was used for experiments. Anti-PY recombinant antibody (RC20) coupled to horseradish peroxidase, rabbit anti-Shc, and anti-Grb2 antibodies were purchased from Transduction Laboratory (Lexington, KY). Rabbit anti-ubiquitin antiserum was purchased from Sigma. Rabbit anti-p85 was a gift from Dr. J. Backer, Albert Einstein College of Medicine. A rabbit anti-Sos antibody that detected both mSos1 and mSos2 was purchased from Santa Cruz Inc. (Santa Cruz, CA). Cy3 coupled donkey anti-goat IgG and fluorescein isothiocyanate (FITC) coupled donkey anti-rabbit-IgG were purchased from Jackson Lab Inc. (Bar Harbor, ME).

Cell Culture and Time Course Study

Cells of the murine macrophage cell line BAC1.2F5 [Morgan et al., 1987] were cultured and stimulated with CSF-1 as described previously [Li et al., 1991; Morgan et al., 1987]. Briefly, cells were incubated in the absence of CSF-1 overnight and stimulated with 13.2 nM human recombinant CSF-1 (a gift from Chiron Corp.) for various times at 4°C or 37°C. Subcellular fractionation by Dounce homogenization and differential centrifugation was performed as previously described [Wang et al., 1996].

Immunoprecipitation and Immunoblotting

Immunoprecipitations from Nonidet-P40 solubilized membrane and cytosol were carried out as described previously [Yeung et al., 1992]. Immunoprecipitated proteins were resolved in a 8–15% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a polyvinylidene difluoride membrane and immunoblotted as described [Yeung et al., 1992]. The membrane was first probed with anti-PY, stripped with 1M NaCl in 10 mM Tris-HCl pH 7.0, then reprobed with other antibodies. Additional reprobing was preceded by stripping with 2% SDS, 1% β-mercaptoethanol, 50 mM Tris-HCl pH 6.8 at 60°C, or with 5% acetic acid at room temperature. For sequential immunoprecipitations, depletion of the antigen was ensured by a repeat immunoprecipitation in which $\leq$ 5% of the antigen was present in the second immunoprecipitate. Supernatants from the second immunoprecipitation were then subjected to immunoprecipitation with the next antibody. For the competition experiments with PY and the proline-rich peptide, immunoprecipitation was carried out as described [Meisner and Czech, 1995], except that PY and/or the proline-rich peptide were added to the cell lysate prior to addition of antibody and were included at the same concentration in the washing buffer.

Immunofluorescence Microscopy

Cells were plated onto chamber slides (21 $\times$ 20 mm well, Lab-Tek, Nalge Nunc, Inc., Naperville, IL) and cultured for at least 24 h. Cells were incubated without CSF-1 for 18 h, then incubated with 13.2 nM CSF-1 at 4°C for 10 min [Li and Stanley, 1991]. The medium was removed and the cells were fixed with 3.7% formaldehyde in buffer F (5 mM PIPES, pH 7.2, 1.1 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 137 mM

KCl, 4.0 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM EGTA, 5.5 mM glucose) for 5 min at 37°C. Subsequent procedures were then carried out at room temperature. The fixed cells were extracted with 0.5% Triton X-100 in buffer F for 10 min and incubated in 0.1 M glycine in buffer F for 10 min to quench aldehyde autofluorescence. After washing five times (5 min each time) with the blocking solution (1% bovine serum albumin; BSA), 5% normal donkey serum, 20 mM Tris-HCl, pH 8.0, 154 mM NaCl, 0.1 mM sodium orthovanadate, 0.02% NaN_3), cells were incubated with the same blocking solution for a further 30 min to block nonspecific immunoreactive sites. Anti-Cbl (10 µg/ml) and anti-CSF-1R (20 µg/ml) antibodies dissolved in blocking solution were incubated with the cells for 1 h. Unbound antibodies were removed by washing the cells five times (5 min each time) with the washing solution (1% BSA, 20 mM Tris-HCl, pH 8.0, 154 mM NaCl). The cells were then incubated for 1 h with donkey anti-goat antibody (3.75 µg/ml, Cy3 labeled) and donkey anti-rabbit antibody (7.5 µg/ml, FITC labeled) dissolved in blocking solution. After thorough washing (five times, 5 min each time) with washing solution, the cells were mounted in a medium containing 50% glycerol, 20 mM Tris-HCl, 154 mM NaCl and 100 mg/ml 1,4-diazabicyclo-[2.2.2]octane (Sigma), and examined under a BioRad MRC 600 Laser Scanning Confocal Microscope (Bio-Rad, Richmond, CA) [Edmonds et al., 1996].

RESULTS

Early CSF-1R Changes in the Response to CSF-1

To study the CSF-1 induced changes in the CSF-1R and the association of the activated CSF-1R with other signaling proteins, we first examined the CSF-1R in cells stimulated with CSF-1 at 37°C. BAC1.2F5 macrophages were incubated with CSF-1 for various times, rapidly cooled to 4°C and fractionated at 4°C into membrane, cytosolic, and nuclear fractions. The CSF-1R was recovered exclusively in the membrane fraction and the kinetics of changes in the receptor and in its association with some other signaling proteins at 37°C are shown in Figure 1 (left panel). Consistent with previous reports [Sengupta et al., 1988; Mori et al., 1995a], at 37°C the CSF-1R undergoes rapid, transient tyrosine phosphorylation and multiubiquitination within the first 3 min of stimulation (Fig. 1A–C). During the same period, the CSF-1R maximally associates with other signaling proteins including Cbl, Sos, p85, p58Shc, and Grb2 (Fig. 1D–H). The coimmunoprecipitation of these proteins is substantially reduced after 3 min when the vast majority of the CSF-1R has been internalized and a significant proportion of the internalized CSF-1R has been dephosphorylated and/or destroyed [Li and Stanley, 1991]. The induced association of Sos, Cbl, and Grb2 appeared to be quite transient (Fig. 1D,E,H). There was persistence of association of the CSF-1R with significant amounts of p58Shc and p85 after 3 min, probably reflecting a steady-state level of association due to the appearance of newly synthesized receptor at the plasma membrane [Guilbert and Stanley, 1986].

To establish the kinetic relationships between the events occurring within the first 3 min of stimulation 37°C, the same changes were studied in cells stimulated with high concentrations of CSF-1 at 4°C. (Fig. 1, right panels). Under these conditions, CSF-1 binds the receptor irreversibly, receptor sites are saturated within 1 min, and there is no internalization of the receptor-ligand complex [Guilbert and Stanley, 1986]. As shown in Figure 1A,B, at 10 min (the earliest time point we can analyze cell fractions from CSF-1 stimulated cells), the CSF-1R became tyrosine phosphorylated, consistent with the earlier demonstration that the 165 kDa CSF-1R species becomes tyrosine phosphorylated within 2 min of stimulation and near maximally tyrosine phosphorylated by 10 min under these conditions [Li and Stanley, 1991; Li et al., 1991]. Furthermore, consistent with the earlier study in which tyrosine phosphorylated CSF-1Rs were shown to be modified between 30 and 90 min after CSF-1 stimulation [Li and Stanley, 1991], a similar effect was observed, commencing at 40 min after CSF-1 addition (Fig. 1B). The mobility of ~50% of ~165 kDa CSF-1R band was decreased to yield CSF-1R bands of higher molecular mass. The proportion of CSF-1Rs involved corresponded to the proportion previously shown to be at the cell surface [Baccarini et al., 1991] and previously shown to be tyrosine phosphorylated [Li et al., 1991] (approximately 50% of the total CSF-1R). The CSF-1R bands of higher molecular mass are tyrosine phosphorylated (Fig. 1), as expected from previous work which also indicated that phosphorylation was not responsible for their reduced mobility [Baccarini et al., 1991]. However, in agreement with the stepwise nature of these changes and the large

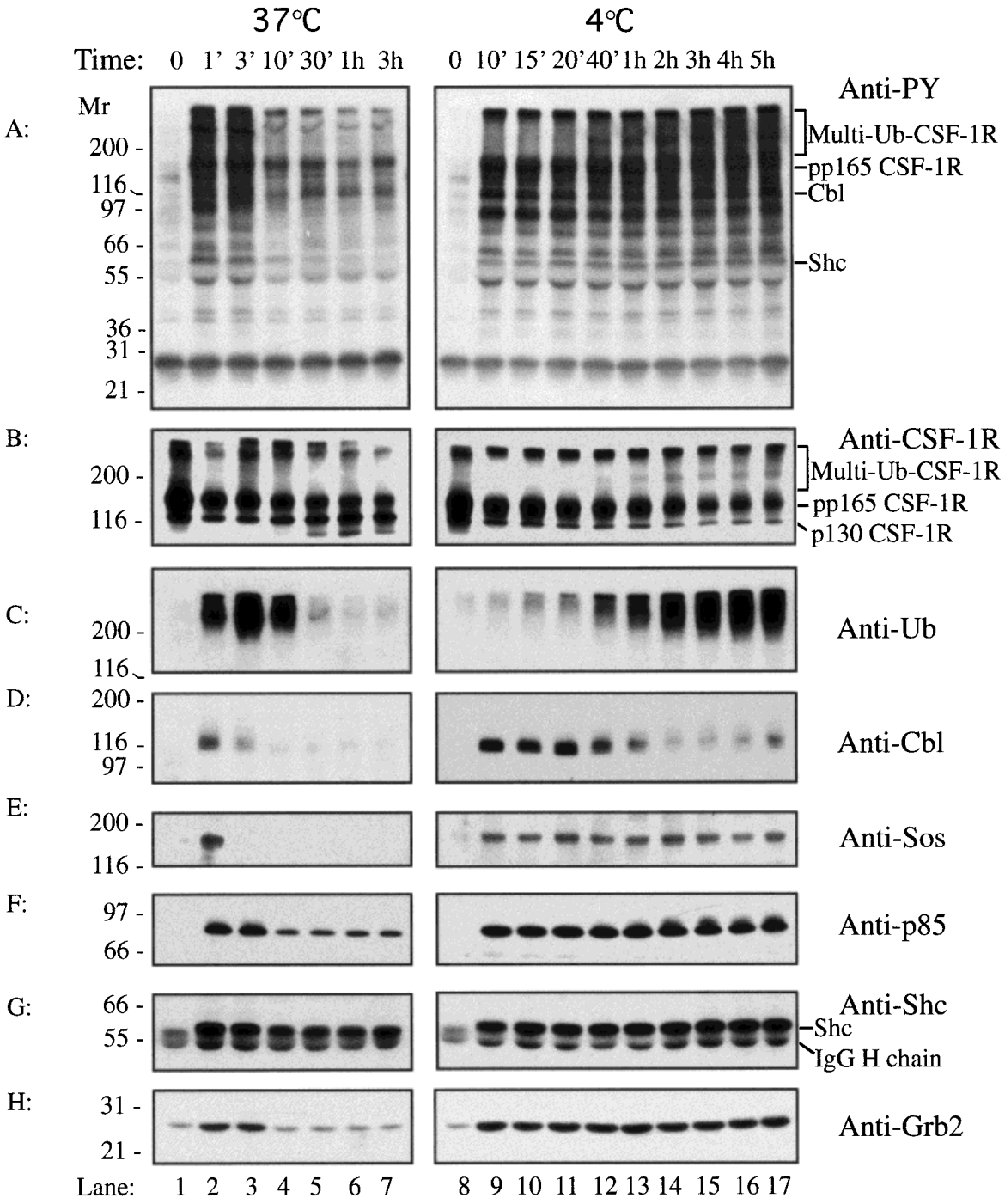

Fig. 1. CSF-1 stimulated CSF-1R phosphorylation, ubiquitination, and association with signaling proteins in BAC1.2F5 macrophage membrane fractions at 37°C and 4°C. Cells were stimulated for the indicated times at 37°C and 4°C. Following subcellular fractionation at 4°C, the Nonidet P-40 solubilized membrane fractions were subjected to anti-CSF-1R immunoprecipitation and the immunoprecipitated proteins resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies. IgG H chain, IgG heavy chain, Ub, ubiquitin. Controls for the multiubiquitination of the CSF-1R included competition with ubiquitin, the use of two additional anti-ubiquitin antibodies and confirmation by mass spectrophotometry (Zhang et al., unpublished communication).

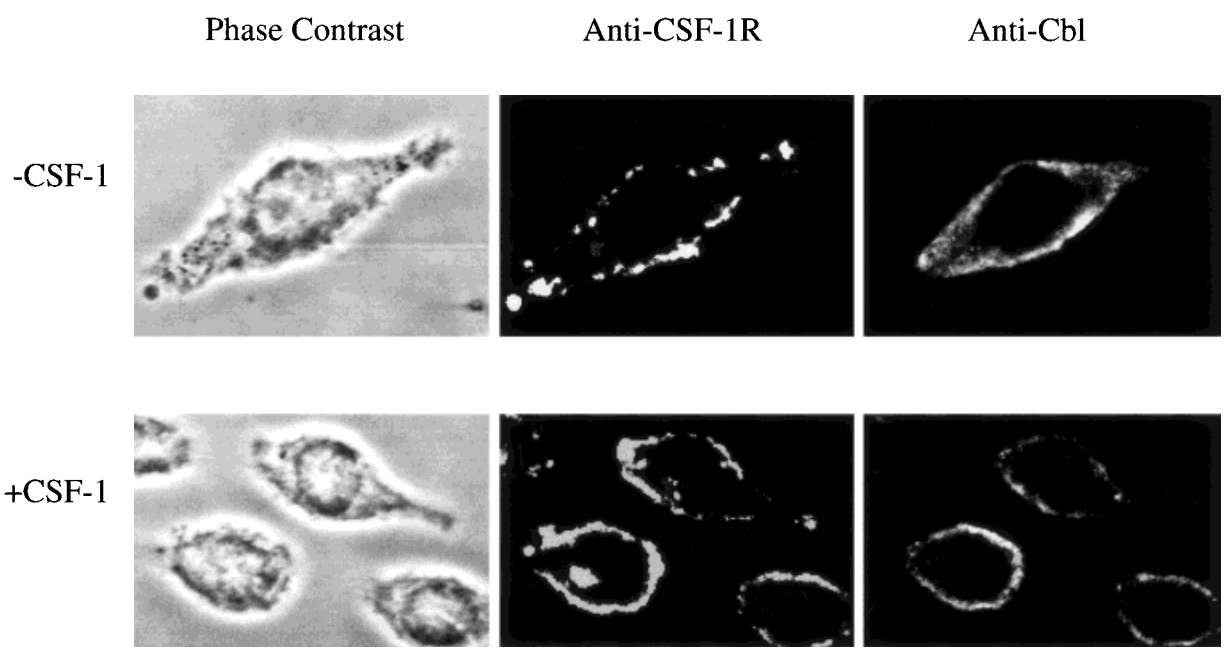

Fig. 2. CSF-1 stimulated translocation of Cbl to the plasma membrane of BAC1.2F5 macrophages at 4°C. Cells were incubated with and without CSF-1 for 10 min at 4°C, fixed, and reacted with anti-CSF-1R or anti-Cbl or control IgG (not shown) prior to reaction with fluorophore-coupled second antibodies, processing, and confocal microscopy as described in the Materials and Methods.

increase in molecular mass observed [Li and Stanley, 1991], these species are ubiquitinated in a pattern consistent with their multiubiquitination (Fig. 1C). Multiubiquitination of the CSF-1R in CSF-1 stimulated cells has been confirmed by mass spectrophotometry of the CSF-1R purified from the membrane fraction of CSF-1 stimulated and unstimulated cells by affinity chromatography and SDS-PAGE [Zhang et al., submitted]. Preceding CSF-1R ubiquitination and within the earliest time point (10 min), the activated and tyrosine phosphorylated CSF-1R associates with Sos, Cbl, p85, p58Shc, and Grb2. CSF-1R association with Sos, p58Shc, Grb2, and at least 50% of p85 initially bound is maintained throughout the time course. By 1 h, coincident with multiubiquitination of the CSF-1R, Cbl association is apparently reduced. However, the dissociation of Cbl may be more apparent than real because of dispersion due to its multiubiquitination and an associated loss of detection by immunoblotting (see below).

CSF-1 Causes Cbl to Move to the Plasma Membrane

Previous studies indicated that CSF-1 causes up to 70–80% of cytosolic Cbl to transiently associate with the membrane fraction of macrophages at 4°C [Wang et al., 1996]. The rapid association of Cbl with the CSF-1R (Fig. 1) following CSF-1 stimulation suggested that Cbl associates with the plasma membrane. To confirm this, the immunolocalization of the CSF-1R and Cbl was analyzed, in macrophages that had been stimulated with CSF-1 for 10 min at 4°C, by confocal microscopy (Fig. 2). In both CSF-1 stimulated and unstimulated cells there was a punctate distribution of the CSF-1R at the cell surface. In addition, some CSF-1R was localized in an internal perinuclear structure, probably the Golgi complex. The Golgi has been shown to contain the CSF-1R precursor which represents ~ 25% of the total cellular CSF-1R as determined by its lower Mr and content of N-linked oligosaccharides of the high mannose type [Rettenmier et al., 1985]. In unstimulated cells, Cbl was found to be distributed throughout the cytoplasm. Following stimulation there was a significant change in its distribution, an increased proportion being associated with the plasma membrane of the cell. These results suggests that the previously reported membrane association of Cbl is with the plasma membrane.

Early Changes in Cbl During the Response to CSF-1

The apparent transient association of Cbl with CSF-1R led us to study Cbl and its associ-

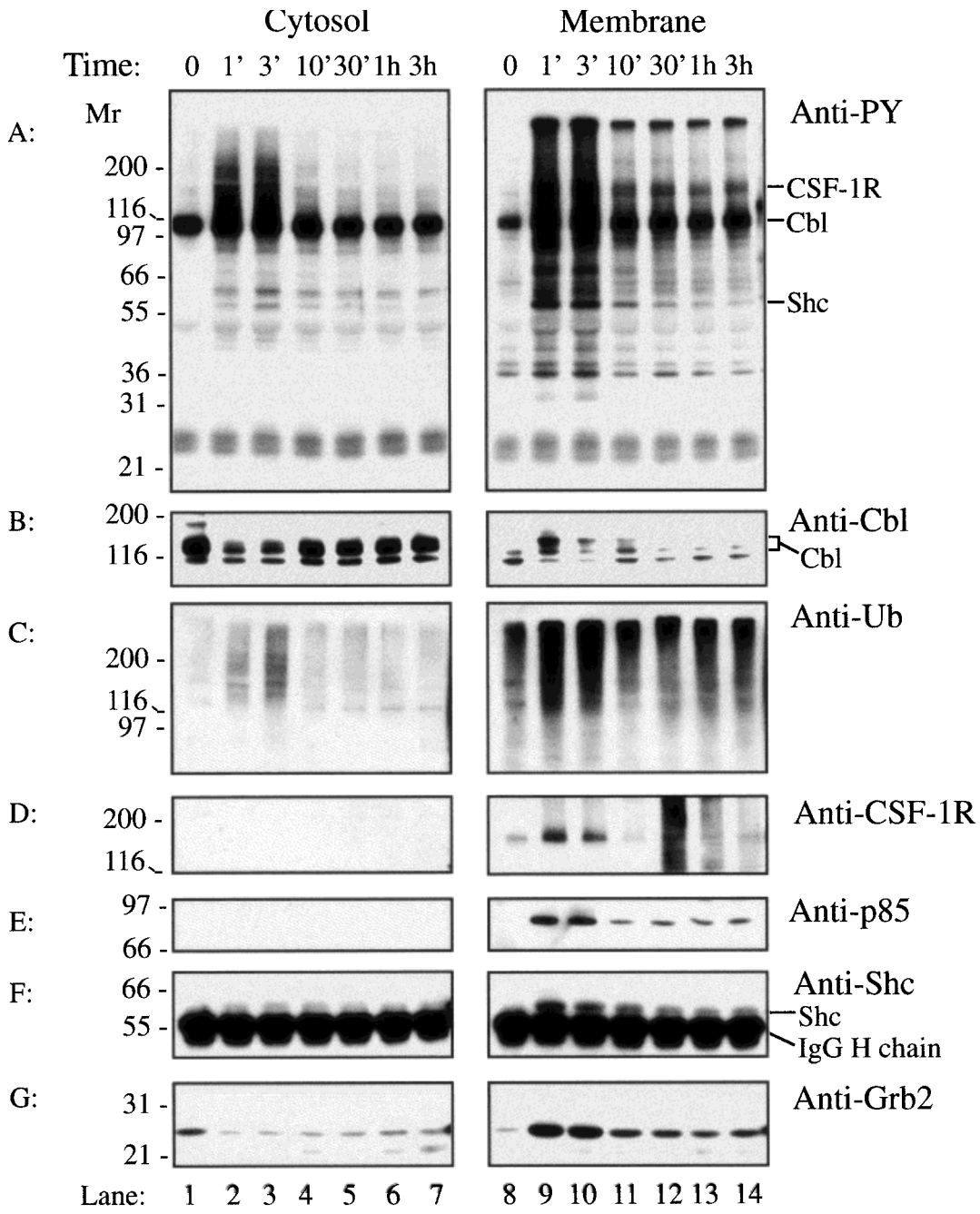

Fig. 3. CSF-1 stimulated Cbl phosphorylation, ubiquitination, and association with signaling proteins in BAC1.2F5 macrophages at 37°C. Cells were stimulated at 37°C for the indicated times. Cytosolic and membrane fractions were subjected to anti-Cbl immunoprecipitation and the immunoprecipitated proteins resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies.

ated proteins under the same conditions. Macrophages were stimulated with CSF-1 at either 37°C (Fig. 3) or 4°C (Fig. 4) over the time course, fractionated into membrane and cytosolic fractions, and Cbl immunoprecipitates of both fractions analyzed by Western blotting. Anti-PY and anti-Cbl Western blots indicate that Cbl is both tyrosine phosphorylated and membrane associated following incubation with CSF-1 for 1 min at 37°C (Fig. 3A,B) or 10 min at 4°C (corresponding to <5 sec at 37°C [Li and Stanley, 1991; Li et al., 1991] (Fig. 4A,B). Commencing at 1 min at 37°C, or 1 h at 4°C , its banding in membrane fraction becomes more

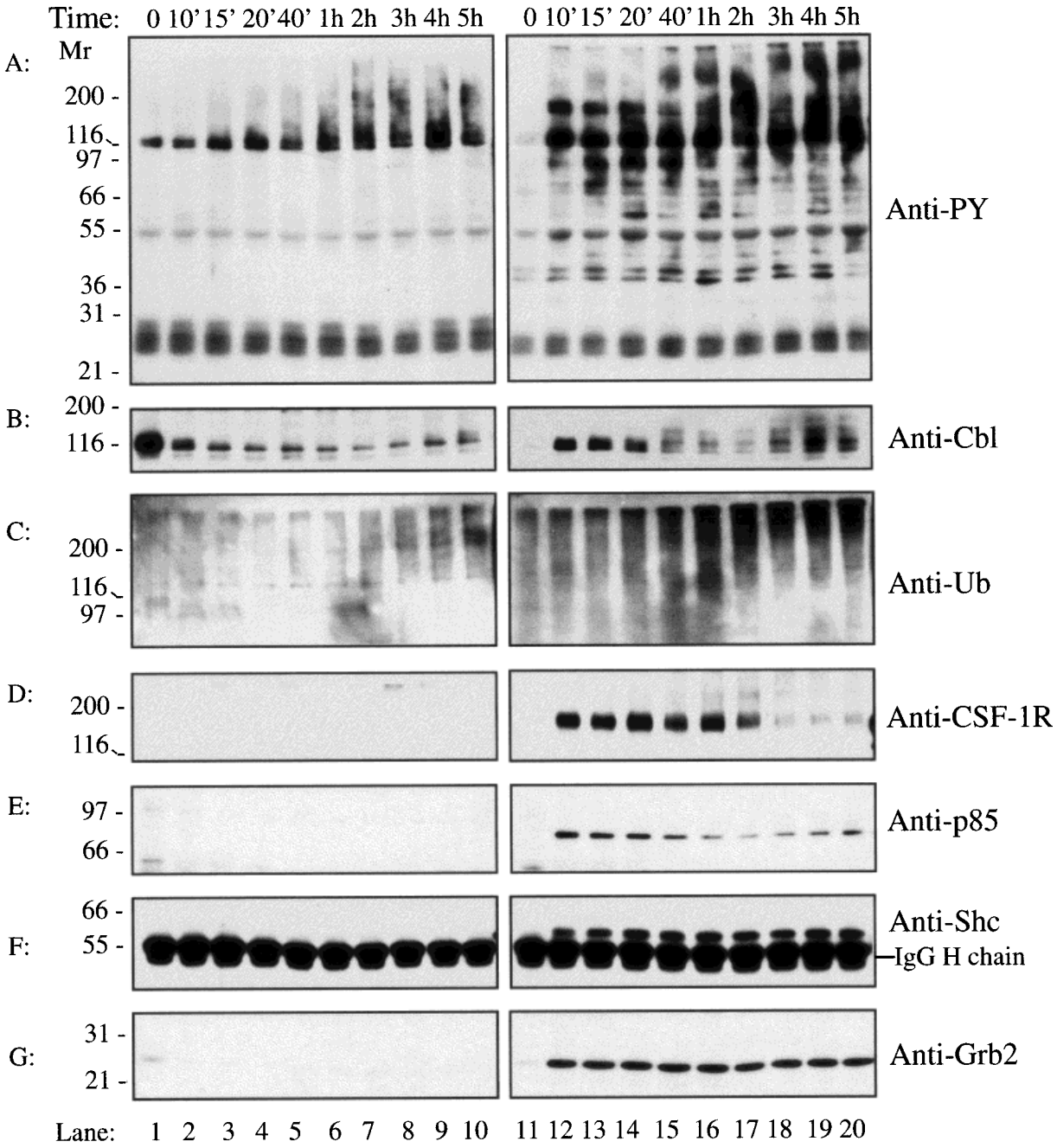

Fig. 4. CSF-1 stimulated Cbl phosphorylation, ubiquitination, and association with signaling proteins in the BAC1.2F5 macrophages at 4°C. Cells were stimulated at 4°C for the indicated times. Cytosolic and membrane fractions were subjected to anti-Cbl immunoprecipitation and the immunoprecipitated proteins resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies.

disperse, or ladder-like, towards higher M_rs and is associated with its poor recovery and visualization on the anti-Cbl Western blots (Figs. 3B, 4B) and with its multiubiquitination [Wang et al., 1996] (Figs. 3C, 4C). Cbl multiubiquitination (Fig. 4C) occurs with similar kinetics to the multiubiquitination of the CSF-1R (Fig. 1C), commencing at 40 min at 4°C. At 4°C, Cbl remains in the membrane fraction in its multiubiquitinated form (Fig. 4B,C). However, by 10 min of stimulation at 37°C, the membrane associated and multiubiquitinated Cbl has returned to the cytoplasm in a deubiquitinated form (Fig. 3B,C). Multiubiquitination of Cbl in

CSF-1 stimulated cells has been confirmed by mass spectrophotometry of Cbl purified from the membrane fraction of CSF-1 stimulated and unstimulated cells by affinity chromatography and SDS-PAGE (Zhang et al., unpublished communication).

Superimposition of the films from the anti-ubiquitin and anti-Cbl western analyses of the membrane fractions of CSF-1 stimulated cells from several experiments, in conjunction with a comparison of the anti-Cbl Western blots of both cytosolic and membrane fractions of both CSF-1 stimulated and unstimulated cells, suggests that at least 90% of the membrane associated Cbl in cells stimulated with CSF-1 for 2 h at 4°C is ubiquinated. Thus, the ubiquination of membrane associated Cbl in CSF-1 stimulated cells is nearly stoichiometric.

Cbl Transiently Associates With Some Proteins and Stably Associates With Others

Cbl is associated with Grb2 in the cytosolic fraction prior to CSF-1 stimulation at both 4°C and 37°C (Figs. 3G, 4G). Immediately following stimulation, Cbl/Cbl-Grb2 disappears from the cytosolic fraction and is recovered in increased amounts in the membrane fraction. Simultaneously with its movement to the membrane, Cbl associates with the CSF-1R, p85, and tyrosine phosphorylated [Wang et al., 1996] p58Shc, as well as with increased amounts of Grb2 (Figs. 3, 4). The Cbl-CSF-1R association is demonstrated in immunoblots of both anti-CSF-1R (Fig. 1) and anti-Cbl (Figs. 3, 4) immunoprecipitates. In contrast, despite its coprecipitation with the CSF-1R (Fig. 1), we were unable to reproducibly demonstrate coprecipitation of Sos in Cbl immunoprecipitates (Fig. 4, see ahead, Fig. 6). Furthermore, anti-Cbl Western blots of anti-Sos immunoprecipitates revealed at least a transient association of Sos with Cbl in the membrane fraction in some instances (Fig. 5), but not others (see ahead, Fig. 6). This lack of reproducibility may be related to transfer of the relatively small amounts of Sos to the membrane upon stimulation (Fig. 5) and the strong preference for coprecipitation of p85, Shc, and the CSF-1R with Cbl over Sos (see ahead, Fig. 6). In contrast to the apparently transient association of Cbl with the CSF-1R at 4°C, its similarly rapid association with tyrosine phosphorylated p58Shc and with Grb2 was sustained over the 5 h time period as was its association with ~50% of the amount of p85 bound initially. Thus kinetic analysis at 4°C suggests an initial association of Cbl, Sos, p85, p58Shc, and Grb2 with the tyrosine phosphorylated and activated CSF-1R, that occurs coincidentally with the tyrosine phosphorylation of Cbl and p58Shc. Subsequently, there is some dissociation of p85 while Sos, p58Shc, and Grb2 association persists (Fig. 1). Cbl apparently dissociates from the CSF-1R at the time of their multiubiquitination, but at 4°C it remains in the membrane fraction. In contrast to the stable association of Sos with the receptor at 4°C (Fig. 1), the association of Sos with Cbl observed in Figure 5D apparently decreases, although this may reflect, as with the CSF-1R Western blot (Fig. 5E), the difficulty of detecting the multiubiquitinated forms of these proteins by western blotting their protein moieties. The association of p58Shc, Grb2, and of ~50% of p85 initially bound with both Cbl (Fig. 4) and Sos (Fig. 5) is stable at 4°C. These studies (Figs. 1, 3–5) indicate that at 4°C, there is significant dissociation of some p85 from the CSF-1R and at the very least, a portion of p85 from both Cbl and Sos at the time of CSF-1R/Cbl multiubiquitination.

By 10 min of stimulation at 37°C, the amounts of Cbl associated CSF-1R, p85, p58Shc, and Grb2 have substantially decreased compared with their association at 3 min. The pattern of association with and dissociation of these molecules from the CSF-1R at 37°C is very similar. In both cases, association with some tyrosine phosphorylated p58Shc and p85 persists until 3 h of stimulation.

Comparison of the Behavior of Sos and Cbl in the Response to CSF-1 at 4°C

In contrast to Cbl, Sos is not detectably tyrosine phosphorylated in response to CSF-1 (Fig 5). While as with Cbl, there is a reduction in cytosolic Sos and an increase in membrane associated Sos following stimulation with CSF-1 (Fig. 5), unlike Cbl, this transfer is not as substantial, nor is Sos multiubiquitinated (Fig. 5, data not shown). Furthermore, while Sos, like Cbl, exhibits an immediate, yet apparently transient association with the CSF-1R, Cbl, and p85 and an immediate and prolonged association with tyrosine phosphorylated p58Shc and Grb2 following stimulation at 4°C (Figs. 4,5D–G), the

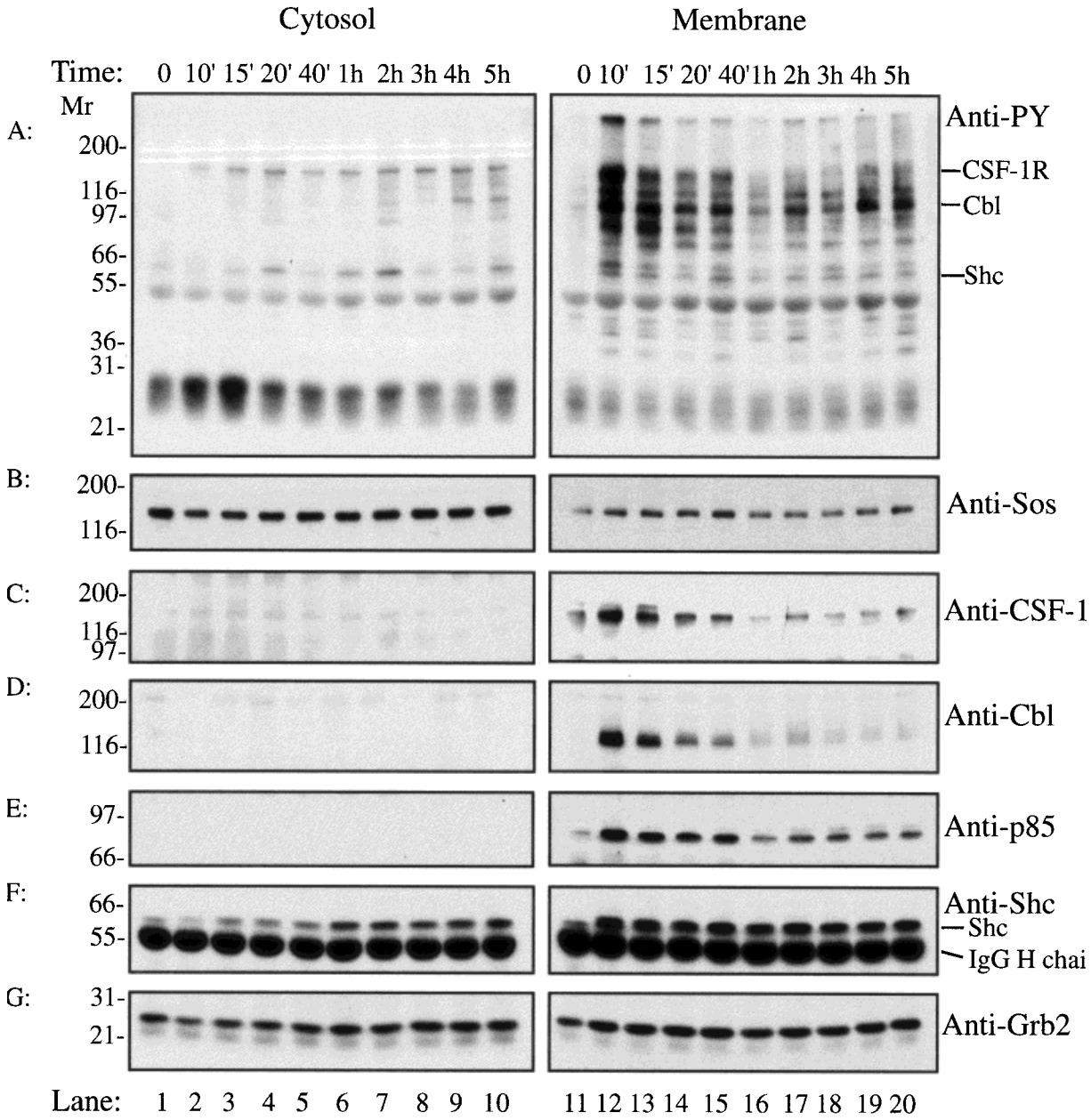

Fig. 5. CSF-1 stimulated Sos association with signaling proteins in BAC1.2F5 macrophages at 4°C. Cells were stimulated at 4°C for the indicated times. Cytosolic and membrane fractions were subjected to anti-Sos immunoprecipitation and the immunoprecipitated proteins resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies.

relative coprecipitation of these molecules by Cbl is much more (see ahead, Fig. 6).

Membrane Complexes Formed by Cbl and Sos are Largely Independent

The foregoing kinetic experiments suggest that Cbl may form several different complexes in cells stimulated with CSF-1. Complexes of a transient nature could involve the CSF-1R, (Sos) and p85, whereas more stable complexes are formed with p58Shc, Grb2, and p85. Both Sos and Cbl form complexes involving the CSF-1R, p85, p58Shc, and Grb2. Despite the association of both Cbl and Sos with the activated CSF-1R, Cbl association with Sos could only be variably demonstrated in Sos immunoprecipitates. To understand whether significant amounts of Cbl or particular Cbl complexes were associated

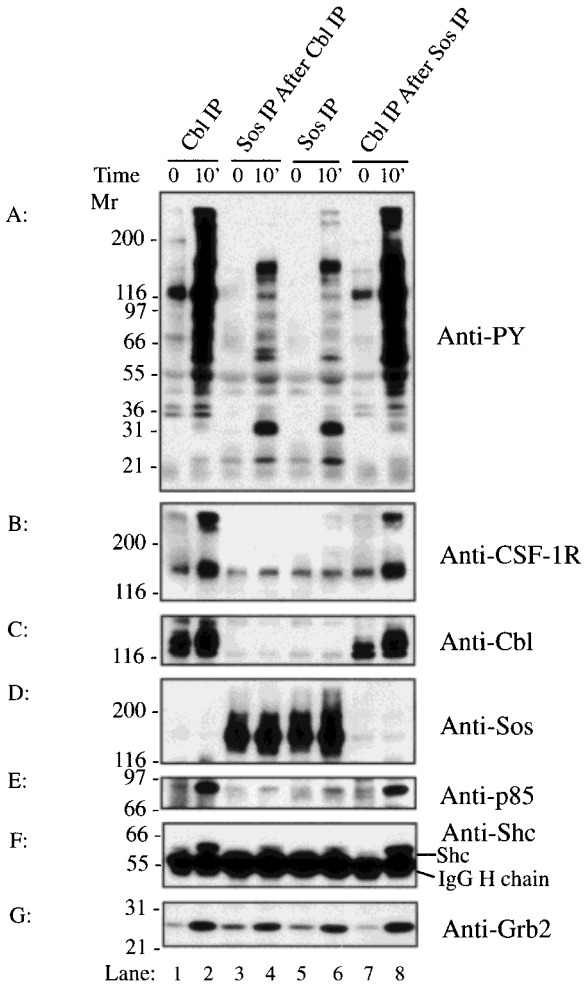

Fig. 6. Cbl and Sos form independent complexes. BAC1.2F5 macrophages were incubated with CSF-1 at 4°C for the indicated times. Solubilized membrane fractions were subjected to anti-Cbl or anti-Sos immunoprecipitation (IP) and the supernatants subsequently immunoprecipitated with anti-Sos or anti-Cbl antibodies. The immunoprecipitated proteins were resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies. The anti-Sos immunoprecipitated 30-kDa and 16-kDa tyrosine phosphorylated bands were more pronounced in this experiment than in others (e.g., Fig. 5).

with Sos, we performed reciprocal immunoprecipitations on Cbl and Sos precleared membrane lysates from cells incubated with CSF-1 for 10 min at 4°C, the time of their maximum association in the response. Only two tyrosine phosphorylated bands, each of > 250 kDa, were removed by anti-Cbl preclearance and there was no alteration in the amounts of CSF-1R, Sos, p85, tyrosine phosphorylated p58Shc, or Grb2 coprecipitated with anti-Sos antibody (Fig. 6). Similarly, the patterns and amounts of proteins coprecipitated with anti-Cbl from Sos precleared and nonprecleared lysates were not significantly different. These results indicate that only very small proportions of Cbl and Sos are likely to be associated with each other in complexes. In particular, however, they indicate that Cbl associates with more tyrosine phosphorylated CSF-1R (and p85 and Shc) than Sos and that the CSF-1R-Sos and CSF-1R-Cbl complexes are independent.

Membrane Complexes of Cbl With Other Tyrosine Phosphorylated Proteins, p85 and Grb2 Involve the CSF-1R

Both the CSF-1R and Cbl form complexes involving a similar set of proteins (CSF-1R, p85, p58Shc, and Grb2). However, Cbl is associated with more tyrosine phosphorylated proteins than Sos (Fig. 6). To examine the relationship between complexes involving the CSF-1R and Cbl, immunoprecipitation of Cbl was carried out on the solubilized membrane fractions with or without CSF-1R preclearance. Immunoprecipitation with Cbl alone resulted in the coprecipitation of many of the proteins immunoprecipitated with the anti-CSF-1R antibody alone (Fig. 7). There was a substantial decrease in the number of co-precipitated tyrosine phosphorylated bands and in the amounts of co-precipitated p85, p58Shc, and Grb2 in Cbl immunoprecipitates of the CSF-1R precleared membrane lysate. These results indicate that Cbl complexes involving these proteins most often include the CSF-1R. Interestingly, however, comparison of the intensity of the anti-Cbl Western blot and the anti-phosphotyrosine Western blot of the Cbl bands indicates that the majority of the membrane associated Cbl is not co-immunoprecipitated with the CSF-1R.

Resistance of Cbl Complexes With the CSF-1R, p85, p58Shc, and Grb2 to Dissociation by Phosphotyrosine, Proline Rich Peptides, or a Combination of Both

Cbl contains both a phosphotyrosine binding domain and several proline-rich regions which have been shown to be involved in binding to SH3 domains of other proteins [Meisner and Czech, 1995]. Using conditions under which there was complete dissociation of the SH2 domain containing phosphatase-1 from a substrate which it bound via its SH2 domains [Berg et al., 1998], we were only able to demonstrate slight dissociation of Cbl from the CSF-1R and p85, and no significant dissociation of Cbl from p58Shc or Grb2 by treatment with phos-

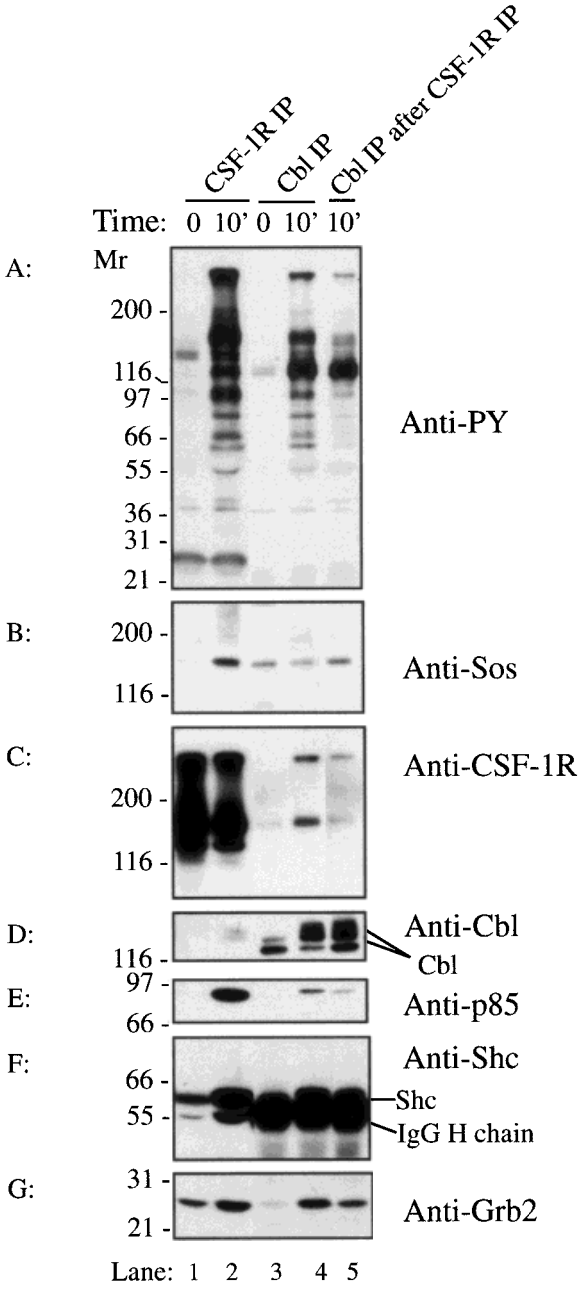

Fig. 7. Membrane complexes of Cbl with other tyrosine phosphorylated proteins, p85 and Grb2, involve the CSF-1R. BAC1.2F5 macrophages were incubated with CSF-1 at 4°C for the indicated times. Solubilized membrane fractions were subjected to anti-Cbl or anti-CSF-1R immunoprecipitation and the supernatants subsequently immunoprecipitated with anti-CSF-1R or anti-Cbl antibodies. The immunoprecipitated proteins were resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies.

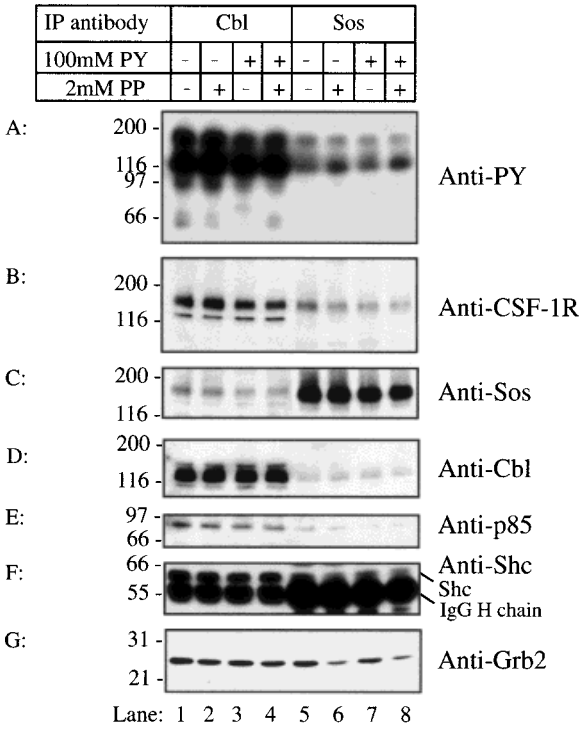

Fig. 8. Phosphotyrosine and a proline-rich peptide fail to dissociate Cbl from complexed signaling proteins. BAC1.2F5 macrophages were stimulated with CSF-1 at 4°C for 10 min. Phosphotyrosine (PY), or a proline-rich peptide (PP) or both were added to solubilized membrane fractions, which were subjected to anti-Cbl immunoprecipitation. The supernatants were subsequently immunoprecipitated with anti-Sos antibody. The immunoprecipitated proteins were resolved in 8–15% SDS-PAGE, transferred, and Western blotted with the indicated antibodies.

photyrosine (Fig. 8). Incubation with a proline-rich peptide, under conditions in which significant dissociation of Grb2 and the CSF-1R from Sos could be demonstrated (Fig. 8, lanes 5–8) and which has previously been shown to cause dissociation of Cbl from the activated EGF receptor and Grb2 in complexes from cell lysates [Meisner and Czech, 1995], failed to cause significant dissociation of Cbl from the CSF-1R, p58Shc, or Grb2 although a slight effect on the dissociation of p85 is apparent (Fig. 8, lane 2). Finally, the effect of the combination of both phosphotyrosine and proline-rich peptides was indistinguishable from the effect of phosphotyrosine alone (Fig. 8, lane 4).

DISCUSSION

Previous studies utilizing chemical crosslinking, ^{32}P and ^{3}H double-labeling and cell surface receptor immunoprecipitation, have described the early CSF-1 induced changes in the CSF-1R in macrophages incubated with CSF-1 [Li and Stanley, 1991; Baccarini et al., 1991]. These studies utilized high concentrations of ligand so that saturation of CSF-1 binding by the recep-

tor was not rate-limiting. Changes consequent to ligand binding included an initial noncovalent CSF-1R dimerization and first wave of CSF-1R tyrosine phosphorylation, tyrosine phosphorylation of cellular proteins, covalent linkage of the CSF-1R dimer subunits, a second wave of CSF-1R tyrosine phosphorylation, and the generation of high molecular weight forms of the CSF-1R. These changes were observed within 1 min of stimulation at 37°C. However, they could be more easily defined at 4°C, because the time frame in which they occurred was extended to approximately 120 min. Both the tyrosine phosphorylation of cell surface CSF-1Rs and their subsequent conversion to higher molecular weight species were shown to be stoichiometric [Li and Stanley, 1991; Li et al., 1991; Baccarini et al., 1991]. Based on these observations and other experiments, it was hypothesized that the tyrosine phosphorylated noncovalent receptor homodimer was the activated, signaling receptor and that the covalent linkage of the extracellular domains of the CSF-1R dimer, the second wave of tyrosine phosphorylation and the increase in CSF-1R molecular weight were preparing the ligand-receptor complex for internalization and/or destruction. The kinetic analyses of the changes in the CSF-1R carried out in the present paper clearly indicate that the previously observed increase in CSF-1R molecular mass [Li and Stanley, 1991] is associated with CSF-1R multiubiquitination. Others have used anti-ubiquitin Western blotting to show that the CSF-1R is multiubiquitinated in the response to CSF-1 [Mori et al., 1995a]. However, our previous experiments [Li and Stanley, 1991; Li et al., 1991; Baccarini et al., 1991], coupled with the data presented here, indicate that CSF-1R multiubiquitination is stoichiometric for cell surface CSF-1Rs. Whether it plays an important role in ligand-induced CSF-1R degradation is not clear.

In a previous study we have shown that CSF-1 stimulation of macrophages leads to the rapid, transient tyrosine phosphorylation of Cbl, its transient association with the membrane fraction and its transient multiubiquitination [Wang et al., 1996]. In the present study, we have demonstrated that the tyrosine phosphorylation of Cbl is coincident with the movement of Cbl to the plasma membrane and its association with the activated tyrosine phosphorylated CSF-1R, p85, p58Shc, and additional Grb2, and precedes its multiubiquitination, which takes place at the membrane simultaneously with the multiubiquitination of the CSF-1R. In contrast to the multubiquitination of the CSF-1R, which precedes CSF-1-CSF-1R complex endocytosis and degradation, the multiubiquitinated Cbl is not destroyed, but is instead rapidly deubiquitinated and returned to the cytoplasm. However, as with the CSF-1R multiubiquitination, the ubiquitination of Cbl in the membrane fraction appears to be nearly stoichiometric. The results of these kinetic studies are consistent with a hypothesis that Cbl targets CSF-1R multiubiquitination. This possibility now seems even more likely in view of the demonstration that Cbl possesses a ubiquitin-associated (UBA) domain at its carboyxterminus (amino acids 844–884). The UBA domain is approximately 55 residues in length, possessing a conserved core region of approximately 45 amino acids [Hofmann and Bucher, 1996]. A number of unrelated proteins either involved in the ubiquitin pathway or possessing regions with homology to ubiquitin itself contain UBA domains. However, most E2 (ubiquitin-conjugating) and E3 (ubiquitin-ligating) enzymes and ubiquitin C-terminal hydrolases do not contain UBA domains, suggesting that the UBA domain does not simply bind ubiquitin or its relatives, but instead confers target specificity to multiple enzymes of the ubiquitination pathway. Thus Cbl, via its UBA domain maybe involved in targeting the ubiquitination system to the CSF-1R. The function of the ubiquitination of either Cbl or the CSF-1R is not clear. In the case of the receptor, it could target the cytoplasmic domain for degradation via the 20S proteosome [Mori et al., 1995b], signal receptor endocytosis [Hicke and Reizman, 1996], signal dissociation of signaling molecules from the CSF-1R or trigger inactivation of the CSF-1R kinase. Our kinetic analysis also raises the possibility that Cbl multiubiquitination leads to the dissociation of Cbl from the CSF-1R and other signaling molecules prior to its return to the cytoplasm. Caution must be exercised, however, in the interpretation of the degree to which Cbl dissociates from the CSF-1R during their multiubiquitination because of the loss of sensitivity of detection of either protein by immunoblotting due to their multiubiquitination-induced dispersion.

The association of Sos with Cbl at best appears to involve a small proportion of each. Although the change in localization and the pattern of association with other proteins in

response to CSF-1 was similar for Cbl (Fig. 4) and Sos (Fig. 5), the association of Sos with these proteins, with the exception of Grb2, was much less (Fig. 6). Despite their strong association with Grb2, sequential immunoprecipitation experiments with Cbl and Sos antibodies (Fig. 6) indicate that Cbl associates with more tyrosine phosphorylated CSF-1R than Sos and that the CSF-1R-Sos and CSF-1R-Cbl complexes are independent. This observation raises the possibility, suggested by others for the EGF receptor [Langdon, 1995], that Cbl-Grb2 competes with Sos-Grb2 for Grb2 binding sites on the CSF-1R. Association solely via these sites is unlikely, however, in view of the limited effect of phosphotyrosine on the dissociation of CSF-1R-Cbl complexes (Fig. 8).

Recent studies of others [Kanagasundaram et al., 1996; Husson et al., 1997] in two different systems indicate that CSF-1 stimulation causes the formation of stable complexes involving the CSF-IR, P13K, CrkII, Grb2, and other tyrosine phosphorylated proteins.

In the CSF-1R preclearance experiments (Fig. 7), a majority of the Cbl in the membrane fraction was not coprecipiated with the CSF-1R, suggesting that either complexes involving the CSF-1R and Cbl are not stable under the conditions used to solubilize the membrane fraction, or that Cbl associates with some other membrane component that is not significantly tyrosine phosphorylated or associated with other tyrosine phosphorylated proteins. However, preclearance of the CSF-1R from the membrane fraction of CSF-1 stimulated cells did result in the removal of Cbl complexes involving Shc, p85, Grb2, and other unidentified tyrosine phosphorylated proteins, indicating that the majority of Cbl in complexes with other PY proteins is associated with the CSF-1R. These complexes, are more substantial than Sos complexes, both in terms of the variety of proteins and their amount (Fig. 6). This observation, together with the coordinate multiubiquitination of Cbl and the CSF-1R, and the presence of the UBA domain at the Cbl C-terminus, suggests that Cbl plays a more complex role than simply negatively regulating Sos function by competing for Sos-Grb2 binding to the CSF-1R. Consistent with this idea, recent studies indicate that there is no alteration in the activation of Ras following EGF receptor activation in cells in which Cbl levels were lowered by antisense oligonucleotide treatment [Ueno et al., 1997].

As a result of this study, several possibilities can be considered for the predicted negative regulatory role of Cbl in CSF-1R signaling, apart from direct effects of Cbl on downstream signaling pathways. The timing of Cbl association with the CSF-1R, their coincident multiubiquitination, and a recent study indicating that multiubiquitination of a truncated form of the yeast mating type receptor, ste2, is involved in its ligand-induced internalization [Hicke and Reizman, 1996] suggest that Cbl could enhance internalization of the CSF-1/CSF-1R complex. In the absence of Cbl, the CSF-1R would therefore be able to signal from the cell surface for a longer time, and certain responses to CSF-1 could be increased. This hypothesis is consistent with the observation that null mutations in *sli-1* have no phenotype in wild type or *let-23*-null backgrounds, but suppress the phenotype of hypomorphic mutations in *let-23* [Jongeward et al., 1995]. Another possibility, suggested by its association in a complex with the CSF-1R and PI3K is that it is somehow involved in targeting the CSF-1R to the lysosome. Mutations in the CSF-1R PI3K-binding domain do not affect receptor internalization, but do inhibit ligand-induced receptor degradation [Carlberg et al., 1991]. Furthermore, experiments with the CSF-1R-related platelet derived growth factor receptor (PDGFR) indicate that wortmannin treatment of cells expressing wild-type PDGFR results in a similar phenotype to those expressing the PDGFR mutant that fails to bind PI3K [Joly et al., 1995]. Cbl may be important for membrane fusion events that deliver the CSF-1R to the lysosome, a fate which is not shared by Cbl, which moves back to the cytoplasm. The present studies provide a framework for future studies of the CSF-1R in cells devoid of Cbl, that should provide important clues to our understanding of Cbl function.

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REFERENCES

Andoniou CE, Thien CBF, Langdon WY. 1994. Tumour induction by activated abl involved tyrosine phosphorylation of the product of the cbl oncogene. EMBO J 13:4515–4523.

Andoniou CE, Thien CB, Langdon WY. 1996. The two major sites of cbl tyrosine phosphorylation in abl-transformed cells select the crkL SH2 domain. Oncogene 12:1981–1989.

Baccarini M, Li W, Dello Sbarba P, Stanley ER. 1991. Increased phosphorylation of the colony stimulating factor-1 receptor following transmembrane signaling. Receptor 1:243–259.

Berg KL, Carlberg K, Rohrschneider LR, Siminovitch KA, Stanley ER. 1998. The major SHP-1-binding, tyrosine-phosphorylated protein in macrophages is a member of the KIR/LIR family and a SHP-1 substrate. Oncogene (in press).

Blake TJ, Shapiro M, Morse III HC, Langdon WY. 1991. The sequences of the human and mouse c-cbl proto-oncogenes show v-cbl was generated by a large truncation encompassing a proline-rich domain and a leucine zipper-like motif. Oncogene 6:653–657.

Brizzi MF, Dentelli P, Lanfrancone L, Rosso A, Pelicci PG, Pegoraro L. 1996. Discrete protein interactions with the Grb2/c-Cbl complex in SCF- and TPO-mediated myeloid cell proliferation. Oncogene 13:2067–2076.

Buday L, Khwaja A, Sipeki S, Faragó A, Downward J. 1996. Interactions of Cbl with two adaptor proteins, Grb2 and Crk, upon T cell activation. J Biol Chem 271:6159–6163.

Byrne PV, Guilbert LJ, Stanley ER. 1981. Distribution of cells bearing receptors for a colony-stimulating factor (CSF) in murine tissues. J Cell Biol 91:848–853.

Büscher D, Dello Sbarba P, Hipskind RA, Rapp UR, Stanley ER, Baccarini M. 1993. v-*raf* confers CSF-1 independent growth to a macrophage cell line and leads to immediate early gene expression without MAP-Kinase activation. Oncogene 8:3323–3332.

Carlberg K, Tapley P, Haystead C, Rohrschneider LR. 1991. The role of kinase activity and the kinase insert region in ligand-induced internalization and degradation of the c-*fms* protein. EMBO J 10:877–883.

Chen ZJ, Parent L, Maniatis T. 1996. Site-specific phosphorylation of IkBa by a novel ubiquitination-dependent protein kinase activity. Cell 84:853–862.

De Jong R, Ten Hoeve J, Heisterkamp N, Groffen J. 1995. Crkl is complexed with tyrosine-phosphorylated Cbl in Ph-positive leukemia. J Biol Chem 270:21468–21471.

Donovan JA, Ota Y, Langdon WY, Samelson LE. 1996. Regulation of the association of $p120^{cbl}$ with Grb2 in Jurkat T Cells. J Biol Chem 271:26369–26374.

Edmonds BT, Wyckoff J, Yeung YG, Wang Y, Stanley ER, Jones J, Segall J and Condeelis J. 1996. Elongation factor-1α is an overexpressed actin binding protein in metastatic mammary adenocarcinoma. J Cell Sci 109: 2705–2714.

Eid H, Chen JH, De Bold AJ. 1995. Regulation of alpha-smooth muscle actin expression in adult cardiomyocytes through a tyrosine kinase signal transduction pathway. Ann NY Acad Sci 752:192–201.

Galisteo ML, Dikic I, Batzer AG, Langdon WY, Schlessinger J. 1995. Tyrosine phosphorylation of the c-*cbl* proto-oncogene protein product and association with epidermal growth factor (EGF) receptor upon EGF stimulation. J Biol Chem 270:20242–20245.

Guilbert LJ, Stanley ER. 1986. The interaction of ^{125}I-colony stimulating factor-1 with bone marrow-derived macrophages. J Biol Chem 261:4024–4032.

Guilbert LJ, Stanley ER. 1980. Specific interaction of murine colony-stimulating factor with mononuclear phagocytic cells. J Cell Biol 85:153–159.

Hamilton JA. 1997. CSF-1 signal transduction. J Leukocyte Biol 62:145–155.

Hicke L, Reizman H. 1996. Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell 84:277–287.

Hochstrasser M. 1996. Protein degradation or regulation: Ub the judge. Cell 84:813–815.

Hofmann K, Bucher P. 1996. The UBA domain: A sequence motif present in multiple enzyme classes of ubiquitination pathway. Trends Biochem Sci 21:172–173.

Husson H, Mograbi B, Schmid-Antomarchi H, Fischer S, Rossi B. 1997. CSF-1 stimulation induces the formation of a multiprotein complex including CSF-1 receptor, c-Cbl, PI 3-kinase, Crk-II and Grb2. Oncogene 14: 2331–2338.

Joly M, Kazlauskas A, Corvera S. 1995. Phosphatidylinositol 3-kinase activity is required at a postendocytic step in platelet-derived growth factor receptor trafficking. J Biol Chem 270:13225–13230.

Jongeward GD, Clandinin TR, Sternberg PW. 1995. *sli-1*, a negative regulator of *let-23*-mediated signaling in *C. elegans*. Genetics 139:1553–1566.

Kanagasundaram V, Jaworowski A, Hamilton JA. 1996. Association between phosphatidylinositol-3 kinase, Cbl and other tyrosine phosphorylated proteins in colony-stimulating factor-1-stimulated macrophages. Biochem J 320:69–77.

Kim TJ, Kim Y-T, Pillai S. 1995. Association of activated phosphatidylinositol 3-kinase with p120cbl in antigen receptor-ligated B cells. J Biol Chem 270:27504–27509.

Langdon WY, Hyland CD, Grumont RJ, Morse HC, III. 1989. The c-*cbl* proto-oncogene is preferentially expressed in thymus and testis tissue and encodes a nuclear protein. J Virol 63:5420–5424.

Langdon WY. 1995. The *cbl* oncogene: a novel substrate of protein tyrosine kinases. Aust NZ J Med 25:859–864.

Li W, Stanley ER. 1991. Role of dimerization and modification of the CSF-1 receptor in its activation and internalization during the CSF-1 response. EMBO J 10:277–288.

Li W, Yeung YG, Stanley ER. 1991. Tyrosine phosphorylation of a common 57-kDa protein in growth factor-stimulated and -transformed cells. J Biol Chem 266: 6808–6814.

Liu YC, Elly C, Yoshida H, Bonnefoy-Berard N, Altman A. 1996. Activation-modulated association of 14–3-3 proteins with Cbl in T cells. J Biol Chem 271:14591–14595.

Meisner H, Conway BR, Hartley D, Czech MP. 1995. Interactions of Cbl with Grb2 and phosphatidylinositol 3'-kinase in activated Jurkat cells. Mol Cell Biol 15: 3571–3578.

Meisner H, Czech MP. 1995. Coupling of the proto-oncogene product c-Cbl to the epidermal growth factor receptor. J Biol Chem 270:25332–25335.

Meisner H, Daga A, Buxton J, Fernandez B, Chawla A, Banerjee U, Czech MP. 1997. Interactions of Drosophila Cbl with epidermal growth factor receptors and role of Cbl in R7 photoreceptor cell development. Mol Cell Biol 17:2217–2225.

Morgan CJ, Pollard JW, Stanley ER. 1987. Isolation and characterization of a cloned growth factor dependent macrophage cell line, BAC12F5. J Cell Physiol 130: 420–427.

Mori S, Claesson-Welsh L, Okuyama Y, Saito Y. 1995a. Ligand-induced polyubiquitination of receptor tyrosine kinases. Biochem Biophys Res Commun 213:32–39.

Mori S, Tanaka K, Omura S, Saito Y. 1995b. Degradation process of ligand-stimulated platelet-derived growth factor β-receptor involves ubiquitin-proteasome proteolytic pathway. J Biol Chem 270:29447–29452.

Ota Y, Beitz LO, Scharenberg AM, Donovan JA, Kinet JP, Samelson LE. 1996. Characterization of Cbl tyrosine phosphorylation and a Cbl-Syk complex in RBL-2H3 cells. J Exp Med 184:1713–1723.

Ota Y, Samelson LE. 1997. The product of the proto-oncogene c-cbl: A negative regulator of the Syk tyrosine kinase. Science 276:418–420.

Paolini R, Kinet J-P. 1993. Cell surface control of the multiubiquitination and deubiquitination of high-affinity immunoglobulin E receptors. EMBO J 12:779–786.

Pollard JW, Stanley ER. 1996. Pleiotropic roles for CSF-1 in development defined by the mouse mutation osteopetrotic (op). Adv Dev Biochem 4:153–193.

Rettenmier CW, Chen JH, Roussel MF, Sherr CJ. 1985. The product of the c-*fms* proto-oncogene: a glycoprotein with associated tyrosine kinase activity. Science 228: 320–322.

Ribon V, Hubbell S, Herrera R, Saltiel AR. 1996. The product of the *cbl* oncogene forms stable complexes in vivo with endogenous Crk in a tyrosine phosphorylation-dependent manner. Mol Cell Biol 16:45–52.

Roussel MF. 1994. Signal transduction by the macrophage-colony-stimulating factor receptor (CSF-1R). J Cell Sci 107 Suppl. 18:105–108.

Sengupta A, Liu W-K, Yeung Y-G, Yeung DC-Y, Frackelton AR, Stanley ER. 1988. Identification and subcellular localization of proteins that are rapidly phosphorylated in tyrosine in response to colony stimulating factor-1. Proc Natl Acad Sci USA 85:8062–8066.

Sherr CJ, Rettenmier CW, Sacca R, Roussel MF, Look AT, Stanley ER. 1985. The c-fms proto-oncogene product is related to the receptor for the mononuclear phagocyte growth factor, CSF-1. Cell 41:665–676.

Smit L, Van der Horst G, Borst J. 1996. Formation of Shc/Grb2- and Crk adaptor complexes containing tyrosine phosphorylated Cbl upon stimulation of the B-cell antigen receptor. Oncogene 13:381–389.

Soltoff SP, Cantley LC. 1996. p120^{cbl} is a cytosolic adapter protein that associates with phosphoinositide 3-kinase in response to epidermal growth factor in PC12 and other cells. J Biol Chem 271:563–567.

Stanley ER. 1994. Colony stimulating factor-1 (macrophage colony stimulating factor). In: Thomson AW, editor. The cytokine handbook. San Diego: Academic Press, pp 387–418.

Tanaka S, Amling M, Neff L, Peyman A, Uhlmann E, Levy JB, Baron R. 1996. c-Cbl is downstream of c-Src in a signalling pathway necessary for bone resorption. Nature 383:528–531.

Thien CBF, Langdon WY. 1997. EGF receptor binding and transformation by v-*cbl* is ablated by the introduction of a loss-of-function mutation from the *Caenorhabditis elegans sli-1* gene. Oncogene 14:2239–2249.

Tsygankov AY, Mahajan S, Fincke JE, Bolen JB. 1996. Specific association of tyrosine-phosphorylated *c*-Cbl with Fyn tyrosine kinase in T cells. J Biol Chem 271: 27130–27137.

Ueno H, Sasaki K, Miyagawa K, Honda H, Mitani K, Yazaki Y, Hirai H. 1997. Antisense repression of proto-oncogene c-Cbl enhances activation of the JAK-STAT pathway but not the Ras pathway in epidermal growth factor receptor signaling. J Biol Chem 272:8739–8743.

Wang Y, Yeung YG, Langdon WY, Stanley ER. 1996. The c-cbl protooncogene product is transiently tyrosine phosphorylated, ubiquitinated and membrane targeted following CSF-1 stimulation of macrophages. J Biol Chem 271:17–20.

Wu RS, Kohn KW, Bonner WM. 1981. Metabolism of ubiquitinated histones. J Biol Chem 256:5916–5920.

Yeung YG, Jubinsky PT, Sengupta A, Yeung DC-Y, Stanley ER. 1987. Purification of the colony-stimulating factor 1 receptor and demonstration of its tyrosine kinase activity. Proc Natl Acad Sci USA 84:1268–1271.

Yeung YG, Berg KL, Pixley FJ, Angeletti RH, Stanley ER. 1992. Protein tyrosine phosphatase-1C is rapidly phosphorylated in tyrosine in macrophages in response to colony stimulating factor-1. J Biol Chem 267: 23447–23450.

Yoon CH, Lee J, Jongeward GD, Sternberg PW. 1995. Similarity of *sli-1*, a regulator of vulval development in *C. elegans*, to the mammalian proto-oncogene c-*cbl*. Science 269:1102–1105.

Zhaung ZP, McCauley R. 1989. Ubiquitin is involved in the in vitro insertion of monoamine oxidase B into mitochondrial outer membranes. J Biol Chem 264:14594–14596.