c-Cbl Associates Directly with the C-terminal Tail of the Receptor for the Macrophage Colony-stimulating Factor, c-Fms, and Down-modulates This Receptor but Not the Viral Oncogene v-Fms*

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The receptor for the macrophage colony-stimulating factor (CSF-1, also termed M-CSF), the tyrosine kinase c-Fms, was originally determined to be the oncogene product of the McDonough strain of feline sarcoma virus, v-Fms. The structural difference between c-Fms and v-Fms amounts to only five point mutations in the extracellular domain, two mutations in the cytoplasmic domain, and the replacement of 50 amino acids by 14 unrelated amino acids at the C-terminal tail. Here, we have identified c-Cbl as the direct binding partner for c-Fms. c-Cbl binds to phosphotyrosine residue 977 at the C-terminal end of feline c-Fms, which is absent in v-Fms. The replacement of the C-terminal end of v-Fms by the corresponding part of c-Fms (vc-Fms) restored the binding potential. As a result, vc-Fms reduced the transforming potency of v-Fms. The overexpression of Cbl did not influence the v-Fms-transformed phenotype, although c-Cbl forms a complex with v-Fms indirectly. In contrast, the expression of Cbl drastically reduced the vc-Fms-transformed phenotype and the activation of Erk and enhanced Fms ubiquitination via phosphotyrosine residue 977. Furthermore, the replacement of tyrosine 977 into phenylalanine in feline c-Fms and vc-Fms reduced the Cbl-dependent ubiquitination. These data suggest that an indirect association of c-Cbl via multimeric complex induced a different signaling pathway from the pathway induced by c-Cbl direct interaction.

In the normal state, binding of growth factors causes dimerization of the receptors and activation of their inherent receptor tyrosine kinases leading to autophosphorylation of the cytoplasmic domains at multiple tyrosine residues. The newly formed phosphotyrosines constitute binding sites for Src homology 2 domain- or phosphotyrosine binding (PTB) domain-containing cytoplasmic proteins, which are thought to participate in the control of mitogenic or differentiation pathways, cell metabolism, and/or cell morphology. After triggering the signal transduction pathways, the receptors are internalized and either recycled again to the cell surface or degraded.

The receptor for the CSF-1, the tyrosine kinase c-Fms, was originally determined to be the oncogene product of the McDonough strain of feline sarcoma virus, v-Fms. The Fms tyrosine kinase was reported to interact with several SH2-domain-containing proteins, including the growth factor receptor bound protein 2 (Grb2; Refs. 2, 3), STAT1 (4), the p85 subunit of phosphatidylinositol (PI) 3-kinase (5), the p120RasGTPase-activating protein (6), phospholipase C-γ (7), c-Src (8), Mona (9), Fms-interacting protein (10), and p55, a polypeptide of yet unknown function (11).

Expression of the viral fms gene in mammalian fibroblasts leads to cell transformation and to formation of fibrosarcoma in vivo. The structural difference between c-Fms and v-Fms consists of only five point mutations in the extracellular domain, two mutations in the cytoplasmic domain, and the replacement of 50 amino acids by 14 unrelated amino acids at the C-terminal tail (12). Roussel et al., (13) showed that a single point mutation at position 301 was solely responsible for a conversion of the human c-fms gene product into a transforming protein. However, Woolford et al. (12) showed that in addition a second mutation involving residue 374 and the exchange of the C-terminal domain were required for an effective transforming potency. The mutation of residues 301 and 374 in the extracellular domain leads to receptor dimerization without a ligand. There is only one tyrosine residue, tyrosine 977, in the feline c-Fms-specific C-terminal tail. Mutation of tyrosine residue 969 of human c-Fms (corresponding to tyrosine 977 of feline c-Fms) slightly enhanced the transforming potency in fibroblasts (14) or gained cytokine independence for growth in the interleukin-3-dependent murine hematopoietic cell line, FDC-P1 (15). Interestingly, the same point mutation has been detected in a number of hematological disorders. The C-terminal mutation has also been observed in children suffering from secondary acute myeloblastic leukemia (AML) or myelodysplasia (16, 17). However, the role of the C-terminal at the molecular level was poorly understood. To elucidate the molecular principles underlying the up-regulation of oncogenic potency via mutation of the C-terminal tail, we employed a yeast two-hybrid screening protocol based on the tyrosine-phosphorylated cytoplasmic domain of c-Fms and v-Fms as baits. We identified

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† The abbreviations used are: CSF, colony-stimulating factor; SH, Src homology; PTB, phosphotyrosine binding; PI, phosphatidylinositol; AML, acute myeloblastic leukemia; GST, glutathione S-transferase.

Retroviral oncogenes have been intensively investigated to further understanding of neoplastic conversion and tumor development in animals (1). A number of receptor tyrosine kinases were originally identified as retroviral oncogene products, including v-Fms, v-erbB, or v-Kit of which the cellular counterparts are receptors for macrophage colony-stimulating factor (CSF-1 or M-CSF), epidermal growth factor, or stem cell factor, respectively. In normal cells, the life span and enzymatic activity of protooncogene-products are tightly regulated. In malignant cells, they escaped from cellular regulatory mechanisms and participated in the control of mitogenic or differentiation pathways, cell metabolism, and/or cell morphology. After triggering the signal transduction pathways, the receptors are internalized and either recycled again to the cell surface or degraded.

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c-Cbl as a binding partner of c-Fms. c-Cbl binds to c-Fms specifically at tyrosine 977, which is absent in v-Fms. Using cells expressing a chimera receptor of v-Fms and c-Fms, we show that the overexpression of c-Cbl suppressed the transforming potential of chimera Fms by the enhancement of the receptor ubiquitination but not that of v-Fms. Furthermore, the replacement of tyrosine residue 977 into phenylalanine avoided the quick ubiquitination of Fms molecules. These data indicate that the observed negative regulatory function of the C-terminal tail is mediated by a Cbl binding site.

MATERIALS AND METHODS

Plasmid Constructions, Transfection, and Yeast Two-hybrid Screening—The construction of LexA fusion genes encoding the cytoplasmic domains of c-Fms, v-Fms, and vc-Fms downstream of LexA using BTM116 vector, the expression in Saccharomyces cerevisiae strain YRN974, and the qualitative and quantitative evaluations of various two-hybrid protein/protein interactions were described previously (3, 10). GST-Cbl-(amino acids 1–350) and Fms-fusion proteins were generated in the pGex system (Amersham Biosciences). For expression in mammalian cells, the feline c-fms, c-fms, and its chimera vc-fms-cDNA were cloned into pcDNA3 (Invitrogen, Carlsbad, CA).

Cells and Antibodies—NIH3T3 and HEK293 cells expressing the v-fms, c-fms, or vc-fms genes were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. CSF-1 (Sigma) was added at a concentration of 2000 units/ml. Rat sera against feline c-Fms and v-Fms were used as described previously (22). Monoclonal antibodies against phosphotyrosine (4G10) and against c-Cbl, ubiquitin, Grb2, and c-Src were from Upstate Biotechnology Incorporated (Lake Placid, NY) or from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit antibodies against ubiquitin and Fms were from Santa Cruz Biotechnology, and Erk1/2 and phospho-Erk1/2 antibodies were from Promega (Madison, WI).

Tyrosine Kinase Assays, Immunoprecipitation, and Immunoblotting—For tyrosine kinase assays, Fms-specific immune complexes were incubated for 20 min at room temperature with 3 μCi of [γ-32P]ATP (Amersham Biosciences) in the presence of 10 nm MnCl2, and were analyzed by SDS-PAGE. For immunoprecipitation studies, 5 × 106 cells were lysed with the lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1% trasyol, 100 μg/ml leupeptin, 1 μg/ml phenylmethylsulfonyl fluoride, 200 μg/ml sodium orthovanadate, 10 mM sodium pyrophosphate, and 100 mM sodium fluoride). For identification by immunoblotting, proteins were transferred onto Polablot polyvinylidene difluoride membrane or nitrocellulose membrane (Macherey-Nagel) by a semi-dry blotting technique. Bound antibody was visualized by incubation of blots in 3 ml of 10% milk in TBS containing 0.1% Tween 20. Detection was performed using ECL reagents (Amersham Biosciences) followed by exposure of the film to X-ray film. All experiments were repeated at least three times with similar results.

RESULTS

Detection of c-Cbl As a c-Fms-specific Direct Binding Partner—Using a yeast two-hybrid technique, we have isolated cDNA encoding several signaling proteins such as PI 3-kinase, Grb2, Grb10, phospholipase C-γ, c-Src, c-Abl, Crk, Fms-interacting protein, and c-Cbl, which specifically interact with the cytoplasmic domain of c-Fms (10). To determine whether one of these associates with c-Fms or v-Fms specifically, we again employed the yeast two-hybrid system using green fluorescent protein as a reporter gene product (3). All of the proteins bound to both v-Fms and c-Fms equally well except the PTB domain of c-Cbl (amino acid residues 1–350). c-Cbl bound to c-Fms but not to v-Fms in this assay system (Fig. 1A). Since the major difference of the cytoplasmic domains of v-Fms and c-Fms is the C-terminal tail, we replaced the last 14 amino acids of v-Fms by 50 amino acids derived from the C-terminal tail of c-Fms (vc-Fms) (Fig. 1B). As shown in Fig. 1, the resultant vc-Fms was indeed able to bind to c-Cbl. As expected, the kinase-negative vc-Fms mutant lacking the ATP-binding site), and 720F-vc-Fms (PI 3-kinase binding site, tyrosine 720, was mutated) (numbers refer to amino acid residues) fusion proteins were co-expressed together with the VP16, the SH2 domain of PI 3-kinase (F 3′K), and the PTB domain of c-Cbl (Chl-PTB) fusion protein in YRN974 (3). Ten thousand cells of four independent transformants were analyzed for fluorescence intensity using a Becton Dickinson FACScan flow cytometer. Values represent the mean values of four independent experiments. B, schematic drawing of the Fms and Fms mutants. JX, juxtamembrane domain; K1, kinase domain 1; K2, kinase domain 2; CT, C-terminal domain.

FIG. 1. c-Cbl binds to c-Fms and chimera Fms (vc-Fms), but not to v-Fms by the yeast two-hybrid assay. A, identifying the c-Fms-specific binding partner by the yeast two-hybrid assay. LexA-c-Fms, v-Fms, vc-Fms (14 amino acids of v-Fms C-terminal tail was replaced by 50 amino acids of c-Fms C-terminal tail), 613M-vc-Fms (a kinase-negative vc-Fms mutant lacking the ATP-binding site), and 720F-vc-Fms (PI 3-kinase binding site, tyrosine 720, was mutated) (numbers refer to amino acid residues) fusion proteins were co-expressed together with the VP16, the SH2 domain of PI 3-kinase (F 3′K), and the PTB domain of c-Cbl (Chl-PTB) fusion protein in YRN974 (3). Ten thousand cells of four independent transformants were analyzed for fluorescence intensity using a Becton Dickinson FACScan flow cytometer. Values represent the mean values of four independent experiments. B, schematic drawing of the Fms and Fms mutants. JX, juxtamembrane domain; K1, kinase domain 1; K2, kinase domain 2; CT, C-terminal domain.
precipitated with anti-Cbl antibody. In this assay, all three, v-, c-, and vc-Fms, were co-immunoprecipitated with c-Cbl molecules (Fig. 2B). In addition, c-Cbl was co-precipitated by anti-Fms antibody with all three autophosphorylated Fms polypeptides. After incubation of autophosphorylated Fms molecules with alkaline phosphatase, the interaction of c-Cbl with Fms was completely abolished (Fig. 2B). Thus, in the presence of cellular proteins, both c- and v-Fms associate with c-Cbl. This is not surprising because it has been well documented that CSF-1 stimulation induces the formation of a multiprotein complex including c-Fms, c-Cbl, CrkII, PI 3-kinase, and Grb2 (19). Here, both Grb2 and PI 3-kinase bind to v-Fms as well. In addition, c-Cbl forms a stable complex via its proline-rich sequence with the SH3 domain of PI 3-kinase and Grb2 (20, 21). Therefore, we utilized the GST fusion protein binding assay using GST-Cbl (amino acid residues 1–350), which lacks the proline-rich domain. In agreement with data obtained from the yeast two-hybrid assay, both 32P-labeled autophosphorylated c-Fms and vc-Fms bound to the GST-Cbl, but v-Fms did not (Fig. 3), suggesting that c-Cbl associates directly with the C-terminal of the c-Fms molecule. However, in addition, c-Cbl binds to v-Fms indirectly through multiprotein complex formation.

The Unique Tyrosine Residue 977 of c-Fms at the C-terminal Tail Provides a Binding Site for c-Cbl—To study whether the c-Cbl/c-Fms interaction relied on the presence of particular phosphotyrosine residues of Fms, we employed a set of GST-Fms fusion proteins including GST-CT-v-Fms (amino acids 904–944), and GST-CT-c-Fms (amino acids 904–980) (Fig. 4A). The recombinant tyrosine-phosphorylated GST-Fms polypeptides were isolated from E. coli strain TKX that expresses an active Elk tyrosine kinase (11). As demonstrated by Western blotting using anti-phosphotyrosine, these proteins were indeed phosphorylated on tyrosine (Fig. 4B). GST Fms fusion proteins were incubated with HEK293 cell extracts, and bound proteins were analyzed by c-Cbl- and Grb2-specific immunoblotting. In agreement with previous data (3), Grb2 bound to both GST-CT-c-Fms and GST-CT-v-Fms that contain the Grb2 binding site, phosphotyrosine 921, while c-Cbl bound to GST-CT-c-Fms exclusively. GST-CT-c-Fms contains two tyrosine residues, tyrosine 921 and tyrosine 977, while GST-CT-v-Fms contains only phosphotyrosine 921 (Fig. 4A). Therefore, we have replaced the unique tyrosine residue in c-Fms, tyrosine 977, by phenylalanine (GST-CT-c-FmsY977F). This GST fusion protein was still able to bind to Grb2; however, c-Cbl was almost undetectable (Fig. 4B). Furthermore, no binding of c-Cbl and Grb2 to unphosphorylated GST fusion proteins

**Fig. 2.** Both v-Fms and c-Fms were immunoprecipitated with c-Cbl in the presence of other signaling molecules. A, v-Fms, c-Fms, and vc-Fms were expressed in HEK293 cells, and cell extracts were used for an *in vitro* kinase reaction in the presence or absence of γ-[32P]ATP. Aliquots of 32P-labeled materials were analyzed by SDS-PAGE and autoradiography. B, 32P-labeled or non-labeled autophosphorylated v-Fms, c-Fms, and vc-Fms were incubated with and without alkaline phosphatase (AP) and were then added to cell lysates from normal HEK293 cells. After incubation, mixtures containing 32P-labeled materials were used for immunoprecipitation with anti-Cbl antibody (IP:c-Cbl) followed by SDS-PAGE and autoradiography, while nonlabeled mixtures were precipitated using anti-Fms antibody (IP:Fms) and analyzed by c-Cbl-specific immunoblot (WB:anti-Cbl).

**Fig. 3.** c-Cbl interacts with Fms and chimera vc-Fms, but not v-Fms in the GST-Cbl-binding assay. GST-Cbl-PTB (amino acids 1–350) or GST alone, were produced in bacterial strains DH5α. v-Fms, c-Fms, and vc-Fms genes were expressed in HEK293 cells, and cell extracts were supplied for *in vitro* kinase reaction in the presence of γ-[32P]ATP. GST-fusion protein or GST (5 μg each) were bound to GT-agarose beads and incubated with 32P-labeled v-Fms, c-Fms, and vc-Fms. Binding materials were analyzed by SDS-PAGE and autoradiography. As a control, aliquots were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue.

**Fig. 4.** Phosphotyrosine 977 at the C-terminal end of c-Fms provides the binding site for c-Cbl. A, a schematic drawing of the GST-Fms fusion proteins: GST, v-CT, GST-CT-v-Fms (amino acids 904–944); c-CT, GST-CT-c-Fms (amino acids 904–980); and 977T/c-CT, GST-CT-c-FmsY977F (amino acids 904–980). B, GST and GST-Fms fusion proteins were produced in bacterial strains DH5α (DH) or TKX (TK) to generate either nonphosphorylated or phosphorylated Fms species, respectively. GST fusion protein or GST (5 μg each) were bound to GT-agarose beads and incubated with HEK293 cell extracts. Binding materials were analyzed by SDS-PAGE followed by immunoblotting (WB) using anti-Cbl or -Grb2 antibodies. As a positive control, HEK293 cell extract was immunoprecipitated with anti-Cbl or -Grb2 (IP). To demonstrate tyrosine phosphorylation of GST-Fms fusion proteins from strain TKX, aliquots of the same samples were analyzed by immunoblotting using an anti-phosphotyrosine antibody (WB: anti-pTyr (anti-pY)). As a control, aliquots were analyzed by SDS-PAGE and staining with Coomassie Brilliant Blue.
were observed (Fig. 4). These data indicate that phosphotyrosine 977 indeed provides the binding site for c-Cbl.

Replacement of the C-terminal of v-Fms by the c-Fms Tail Reduced the Transforming Potency of v-Fms—One important characteristic which distinguishes normal cells from transformed cells is the ability of the latter to form colonies in soft agar. To determine whether the replacement of the C-terminal tail influences the transforming potential of v-Fms, $5 \times 10^5$ NIH3T3 cells were transfected with 10 μg of pcDNA3 containing feline c-Fms, v-Fms, and chimera vc-Fms. Fig. 5 shows that v-Fms transfectants formed colonies in 0.5% soft agar, but c-Fms transfectants were not able to form colonies. In agreement with previous data (12), vc-Fms transfectants formed colonies; however, sizes of colonies were drastically reduced.

Overexpression of c-Cbl Abolished the Transforming Potential of vc-Fms but Not v-Fms—To determine whether the observed reduction of v-Fms-transforming potency by the C-terminal replacement is due to a direct interaction with c-Cbl, we introduced the exogenous c-Cbl at different concentrations into vc-Fms- and v-Fms-transformed cells. For this experiment, we further established cell lines that express vc-Fms or v-Fms. Established cell lines were transfected with 1 and 9 μg of pcDNA3 containing c-Cbl cDNA and tested again for formation of colonies in soft agar. In the absence of exogenous c-Cbl, v-Fms-transformed cells formed colonies with an average size of 60 μm in diameter, while vc-Fms-transformed cells formed colonies with an average size of 35 μm in diameter within 6 days (Fig. 6). Sizes of colonies of vc-Fms expressing cells were drastically reduced by overexpression of exogenous c-Cbl after transfection even with 1 μg of pcDNA3 containing c-Cbl cDNA. In contrast, c-Cbl expression (Fig. 6A, 1 μg) did not change, if rather enhanced, the size of colonies after the same treatment of v-Fms-transformed cells. These results indicate that the direct interaction with c-Cbl to the Fms molecule may downmodulate the Fms signal. In addition, although wild-type v-Fms interacts with c-Cbl indirectly via other signaling molecules such as PI 3-kinase and Grb2, c-Cbl did not affect transforming potency of v-Fms, suggesting an indirect association of c-Cbl via a multimeric complex that induced a different signaling pathway from the pathway induced by c-Cbl direct interaction.

V-Fms and vc-Fms Were Ubiquitinated in the Tyrosine Residue 977 and c-Cbl-dependent Manner—Since it has been well documented that c-Cbl plays a role in protein ubiquitination, the question arises as to whether the reduced transforming potency of vc-Fms is a result of enhanced ubiquitination. Therefore, we compared first the ubiquitination of v-Fms and vc-Fms upon stimulation with CSF-1. Sister cultures of $1 \times 10^6$ cells each derived from v- or vc-Fms-transformed 3T3 cell lines were labeled with $[^{3}H]$leucin for 16 h and were stimulated with CSF-1 for 3, 10, or 30 min. Cell extracts from each preparation were supplied for Fms-specific immunoprecipitation. In agreement with previous data (22), about 10% of v-Fms molecules were detected as a mature glycoprotein, gp140+$\text{Fms}$ that is expressed at the cell surface. Although similar amounts of immature glycoprotein gp120+$\text{Fms}$ and gp130+vc-Fms were detected from both cell lines, less of the mature glycoprotein of vc-Fms, gp150+vc-Fms, was detected than gp140+$\text{Fms}$ (Fig. 7A). The same aliquots of Fms-specific immunoprecipitates were analyzed by ubiquitin-specific immunoblot. vc-Fms was ubiquitinated about 2-fold more than v-Fms; however, no significant difference was observed in the presence or absence of CSF-1. It is noteworthy that vc-Fms was dimerized and activated constitutively without CSF-1 via mutations in the extracellular domain (12, 13) and might be ubiquitinated constantly to some extent. As control, cell extracts from the same preparation were analyzed by Western blot using anti-Erk and activated Erk antibodies. In both cell lines, Erk1/2 was activated slightly without ligand; however, CSF-1 stimulation induced an activation of Erk within 3 min in both cell lines, whereby the level of phosphorylation decreased within 10 min. To determine whether c-Cbl molecules indeed influenced ubiquitination via phospho-Tyr-977, we generated the mutant vc-Fms whose tyrosine 977 was replaced by phenylalanine (vc-Y977F-Fms). Five μg of each vc-Fms and vc-Y977F-Fms cDNA were transfected into 2 $\times 10^5$ HEK293 cells with and without c-Cbl cDNA. After serum starvation for 16 h, cells were stimulated with CSF-1 for 3 or 30 min at 37 °C. Aliquots of cell extracts were immunoprecipitated by Fms-specific antibodies and were analyzed by Fms- or ubiquitin-specific immunoblotting. The following results were obtained. Firstly, c-Cbl over-expression down-modulated Erk activation induced by CSF-1 in vc-Fms-transformed cells.
The over-expression of c-Cbl in vc-Fms-suppressed Erk activation and enhanced vc-Fms ubiquitination via a direct c-Cbl binding site, tyrosine 977. A, 1 x 10⁶ cells of vc-Fms- and vc-Fms-expressing NIH3T3 cell lines were labeled with [³H]leucine for 16 h and then stimulated with CSF-1 for 10 or 30 min. Cell extracts were immunoprecipitated with anti-v-Fms rat antibody. Precipitates were divided into two aliquots: one was precipitated by anti-v-Fms rat antibody (IP:Fms) followed by ubiquitin-specific immunoblot (WB:anti-Ub) and the other cell lysates (WCL) were analyzed by c-Cbl, phosphoErk, or Erk-specific immunoblot (WB). B, five µg of pcDNA3 containing vc-Fms or vc-Y977F-Fms cDNA were transfected into 2 x 10⁵ of HEK293 together with or without 5 µg of c-Cbl cDNA. After 36 h of transfection, cells were serum-starved for 16 h and then stimulated with CSF-1 for 3 or 30 min at 37 °C. Cell extracts were divided into two aliquots and precipitated by anti-Fms rat antibody (IP:Fms) followed by ubiquitin (WB:anti-Ub) or Fms-specific immunoblot (WB:anti-Fms), or cell lysates (WCL) were analyzed by c-Cbl, phosphoErk, or Erk-specific immunoblot (WB).

Fig. 7. The over-expression of c-Cbl in vc-Fms-suppressed the ubiquitination. Five µg of pcDNA3 containing c-Fms or c-Y977F-Fms cDNA were transfected into 2 x 10⁵ of HEK293 together with or without 5 µg of c-Cbl cDNA. After 36 h of transfection, cells were serum-starved for 16 h and were then stimulated with CSF-1 for 3 or 30 min at 37 °C. Cell extracts were divided into two aliquots and precipitated by anti-Fms rat antibody (IP:Fms) followed by ubiquitin (WB:anti-Ub) or Fms-specific immunoblot (WB:anti-Fms), or cell lysates (WCL) were analyzed by c-Cbl, phosphoErk, or Erk-specific immunoblot (WB).

Role of c-Cbl in Fms Tyrosine Kinase-mediated Cell Transformation

Employing a yeast two-hybrid technique, we detected c-Cbl as a direct interacting partner of c-Fms tyrosine kinase. We show here c-Cbl binds to phosphotyrosine 977 at the C-terminal tail of feline c-Fms, which is absent in the v-Fms molecule. Oncogenic v-Fms, which was reconstituted the Cbl binding site, did not transform cells in the presence of the high level of c-Cbl, indicating that the direct binding of c-Cbl plays a key role in Fms tyrosine kinase-mediated cell transformation. Furthermore, the exogenous c-Cbl expression in vc-Y977F-Fms cells did not influence Fms ubiquitination of and Erk activation mediated by CSF-1. Interestingly, v-Fms formed a complex with c-Cbl indirectly; however, expression of c-Cbl did not alter transforming potency of v-Fms, indicating that c-Cbl may mediate two different signaling pathways dependent upon distinct multimeric complexes.

The protooncogene c-cbl product is the 120-kDa protein containing an unconventional PTB domain, a ring finger, a proline rich-domain, and a leucine zipper-like domain. This molecule was originally identified as a retroviral oncogene product, v-Cbl (24), that contains only the PTB domain of c-Cbl. Recently, it was demonstrated that the protooncogene product c-Cbl acts as a ubiquitin ligase, E3 ubiquitin conjugate enzyme, (25–27) and leads to the increased rate of ubiquitination and degradation of several receptor tyrosine kinases, including the receptor for epidermal growth factor, platelet-derived growth factor, and CSF-1 (23, 26–29).

We show here for the first time that c-Cbl binds to c-Cbl directly via phosphotyrosine 977. It has been suggested that Cbl is associated with phosphotyrosine 723 in human c-Fms (corresponding to the tyrosine residue 720 in v-Fms and feline c-Fms). Since this tyrosine residue was identified as a PI 3-kinase binding site, this interaction may be via PI 3-kinase (18). The authors showed that c-Cbl molecules were less immunoprecipitated by a mutant human c-Fms in which tyrosine 723 was changed to phenylalanine. In agreement with this work,
we observed that the phosphorylated v-Fms formed a complex with c-Cbl in the presence of other signaling molecules, indicating that c-Cbl binds also indirectly via a multiprotein complex. Furthermore, it has been demonstrated that PI 3-kinase or Grb2 forms a complex with c-Cbl upon stimulation with CSF-1 (30). Taken together these data suggest that the Fms/c-Cbl interaction may mediate two different signaling pathways dependent upon distinct multimeric complexes; direct c-Cbl/Fms association leads to the down-modulation of receptor signaling, whereas the indirect association via a multiprotein complex leads instead to the up-regulation of receptor signaling. Along these same lines, Oberg et al. (31) reported that the mutation of the PI 3-kinase binding site, tyrosine 731, in the cbl molecule down-regulated Lyn tyrosine kinase-mediated thymidine incorporation, suggesting that the association of Cbl with PI 3-kinase plays a key role in proliferative signaling pathways. It has also been shown that the interaction of PI 3-kinase with c-Cbl enhanced PI 3-kinase activity (32), suggesting that c-Cbl may act as a positive regulator for signaling by the activation of receptor tyrosine kinase. In addition, c-Cbl also up-regulates Fc receptor-mediated platelet activation (33) or CD16-mediated signaling (34) via distinct multiprotein complexes. On the other hand, it is clear that the direct binding of c-Cbl to phosphotyrosine 977 of Fms leads to the down-modulation of Fms and its downstream signal cascades. Interestingly, the ubiquitination of platelet-derived growth factor β receptor, which is closely related to Fms tyrosine kinase, was drastically reduced via deletion of 98 amino acids at the C-terminal end or replacement of tyrosine residues 1009 and 1021 by phenylalanine at the C-terminal domain. Furthermore, the ubiquitination-deficient receptors possessed an amplified mitogenic activity (35). The C-terminal tail of Neu receptor tyrosine kinase is also required for c-Cbl-mediated ubiquitination (36) and mutations that impair the property of c-Cbl to induce the ubiquitination of the epidermal growth factor receptor have oncogenic properties (25), suggesting that C-terminal tail has a function as a regulatory domain for many receptor tyrosine kinases. These data also reveal that receptor ubiquitination is one of the important control mechanisms for anti-cell transformation. Interestingly, impairing the function of TSG101/Vsp23, a protein that contains an inactive E2 ubiquitin-conjugase domain, perturbs endosomal trafficking and induces cell transformation (37).

In addition to the Cbl binding site, the C-terminal tail of c-Fms contains a PEST-like domain of 33 amino acids (amino acid residues 925–957 of feline c-Fms) with 18 serine residues, five glutamate residues, two proline residues, and one threonine residue. Hence, Oberg et al. (38) reported that Notch 1 is ubiquitinated by mSel-10 and that ubiquitination requires the presence of the Notch 1 C-terminal domain, including the PEST domain. The role of this PEST-like domain of c-Fms, however, still remains to be studied.

It is noteworthy that mutation of the human c-fms gene at codon 969 (corresponding to the feline 977 tyrosine residue) has been detected in a number of hematological disorders, AML, B-cell lymphoma and children suffering from secondary AML or myelodysplasia (16, 17). These observations taken together, indicate that this mutation may represent a general mechanism by which oncogenic potential is achieved by escaping the c-Cbl, negative regulation of tyrosine kinase signaling.

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