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Association of Cbl with Fms and p85 in response to macrophage colony-stimulating factor

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Abstract Tyrosine phosphorylation of Cbl and its association with signal-transducing molecules in response to macrophage colony-stimulating factor (M-CSF) were analyzed by using cell lines which express the wild-type and a mutant M-CSF receptor, Fms. We found that in a clone, F723 TF-1 cells expressing mutant Fms in which tyrosine 723 had been substituted with phenylalanine, the M-CSF stimulation-dependent association between Cbl and Fms was markedly impaired. However, phosphorylation of Cbl and its association with the p85 subunit of phosphatidylinositol 3-kinase were induced in these mutant cells as seen in the wild-type fms transfectant. These results suggest that phosphorylation of tyrosine 723 is particularly important for the recruitment of Cbl to the M-CSF receptor, but is not required for the phosphorylation and binding of Cbl to signal-transducing molecules such as p85.

Key words: Macrophage colony-stimulating factor; Fms; Cbl; p85

1. Introduction

Macrophage colony-stimulating factor (M-CSF) is required for the proliferation, survival, differentiation and activation of cells of monocyte/macrophage lineage [1,2]. The receptor for M-CSF (Fms) is encoded by the c-fms proto-oncogene, and expressed on cells of the monocyte/macrophage lineage including osteoclasts and trophoblastic cells [3,4]. The receptor contains a cytoplasmic tyrosine kinase domain split by the kinase insert region. M-CSF binding results in the dimerization of Fms followed by the activation of its intrinsic tyrosine kinase [5]. Many cellular proteins, including Fms itself, are phosphorylated at tyrosine by the activation of Fms. Three tyrosine autophosphorylation sites have been identified within the kinase insert region in human Fms, 699, 708 and 723 [6,7]. When these tyrosines are phosphorylated, a set of signaling molecules containing Src homology (SH) 2 domains are recruited to these sites, which then activate respective signal transduction pathways. Grb2 binds to phosphorylated tyrosine at 699 [8] and may activate the Ras pathway through association with the guanine nucleotide exchanging factor, Sos-1 [9,10]. The p85 subunit of phosphatidylinositol 3-kinase (PI3-kinase) binds to phosphorylated tyrosine at 723 [7,11], thereby increasing the catalytic activity of the p110 subunit [12]. Recent findings suggested that phospholipase C (PLC)-γ2 also binds to this site [13]. Tyrosine 708 has been suggested to be the binding site for STAT1 [14]. There are some additional phosphorylation sites that are important for M-CSF signaling. The phosphorylation of tyrosine 809 is responsible for the full activation of the tyrosine kinase of Fms or cell surface expression of Mac2 in response to M-CSF stimulation [15,16]. Tyrosine 809 also functions in the activation of STAT proteins or the induction of c-myc and cyclin D1 mRNAs in response to M-CSF stimulation [17–19]. Tyrosine 561 is suggested to be the binding site for Src family protein kinases [20]. Src activation is also important for the M-CSF-dependent induction of c-myc and DNA synthesis [21,22].

The proto-oncogene product Cbl is a 120 kDa protein with a phosphotyrosine binding (PTB) domain, a RING finger motif, a highly basic region, a proline rich region (SH3 binding domain), and a putative leucine zipper motif [23,24]. A variety of stimulants such as growth factors or interleukins induce the tyrosine phosphorylation of Cbl which becomes associated with various proteins containing SH2 and SH3 such as protein tyrosine kinases, p85 subunit of PI3-kinase, Crk and 14-3-3 [25].

In M-CSF signaling, Cbl was associated with Grb2 in the unstimulated state, and phosphorylated and membrane-targeted following M-CSF stimulation in the macrophage cell line, BAC1.2F5 [26]. In this same cell line, Cbl was associated with p85 after M-CSF stimulation [27]. An association between Cbl and Fms has been reported in the murine macrophage cell line P388 [28]. However, it is not clear how this molecule can be recruited to the M-CSF receptor. As mentioned above, Grb2 and p85 were shown to bind to phosphorylated tyrosine at 699 and 723 in Fms respectively. Therefore, it must be clarified whether these proteins play the role of adapter between Cbl and Fms, and whether phosphorylation of these tyrosines is responsible for these interactions or not.

In this study, we transfected c-fms cDNA into the human myeloid cell line, TF-1, and established cell lines overexpressing wild-type or a series of tyrosine-phenylalanine mutants of Fms by which association of p85 and Grb2 with Fms was impaired. By using these clones, we analyzed behaviors of Cbl such as tyrosine phosphorylation and its association with Fms and p85 upon M-CSF stimulation.

2. Materials and methods

2.1. Cells

To obtain bone marrow macrophages, the precursor cells in bone marrow of 8-week-old male C57BL/6 mice were cultured for 5 days

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with RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone Laboratories, Logan, UT, USA) and with 100 ng/ml of human recombinant M-CSF [29]. Then, adherent cells were recovered and used for experiments.

c-fms expression plasmids were constructed and transfected into the human erythroleukemic cell line TF-1 [30] as follows: elongation factor promoter was separated from pEF-BOS [31] and introduced into the expression vector pRcCMV (Invitrogen, San Diego, CA, USA). Human c-fms gene (kindly provided by Dr. C.J. Sherr) was inserted downstream of the elongation factor promoter in this plasmid. We substituted each tyrosine at 561, 699, 723 and 809 with phenylalanine in Fms by means of PCR-based site-directed mutagenesis as described [32]. The following oligonucleotides were used as PCR mutation primers: 5′-TGAGGGCAACAGTTTTACTT-3′, 5′-CGTCGACTTTAAGAACA-3′, 5′-GGACACCTTTGTGGAGA-3′ and 5′-CTCCAACTTCATTGTCA-3′ for 561, 699, 723 and 809, respectively. The mutation was verified by DNA sequencing. The expression plasmids were transfected into TF-1 using Lipofectin methods (Gibco BRL, Grand Island, NY, USA). Cells were selected in G418 (Geneticin, Gibco BRL) to establish cell lines. Clones expressing high levels of the gene were selected by means of flow cytometry using an anti-c-fms antibody (Ab2, Oncogene Science, Uniondale, NY, USA). The transfectant of vector alone in TF-1 cells (MOCK) and stable TF-1 cell lines over-expressing wild-type (WT) or mutant (F561, F699, F723 and F809) human Fms were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (Hyclone Laboratories) and with 5 ng/ml of human recombinant GM-CSF (kindly provided by the Kirin Brewery, Tokyo, Japan).

2.2. Immunoprecipitation and immunoblotting

Cells were serum-starved for 4 h, then left untreated or stimulated for 3 min at 37°C with M-CSF (100 ng/ml). The cells were lysed in ice-cold TNE (10 mM Tris–HCl pH 7.8, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 20 mg/ml aprotinin, 2 mM Na_3VO_4). Cell lysates were pre-cleared with protein G-Sepharose (Sigma, St. Louis, MO, USA) for 1 h, then incubated with the appropriate antibodies for 4 h at 4°C. The immunocomplexes were recovered with protein G-Sepharose and washed with TNE lysis buffer. The precipitates were separated by 8% acrylamide SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a PVDF membrane (Immobilon, Millipore, Bedford, MA, USA). The membrane was immunoblotted with appropriate antibodies, then visualized by enhanced chemiluminescence (Dupont NEN, Boston, MA, USA). Anti-phosphotyrosine (4G10), anti-Fms and anti-p85 subunit of PI3-kinase antibodies were obtained from UBI (Lake Placid, NY, USA). Anti-Cbl (C-15), anti-CrkL (C-20) and anti-GST (B-14) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Fms antibody (Ab2, Oncogene Science) was used for immunoprecipitation. Anti-Cbl antibody (Transduction Laboratory, Lexington, KY, USA) was used for immunoblotting.

3. Results

3.1. $p120^{Cbl}$ is one of the major substrates of tyrosine kinase activated by M-CSF stimulation

Bone marrow-derived macrophages were stimulated by M-CSF and the tyrosine-phosphorylated proteins were analyzed by immunoblotting with anti-phosphotyrosine antibody. As reported previously [28], Cbl was identified as one of the prominent substrates of the tyrosine kinase activated by M-CSF stimulation (Fig. 1A). In an M-CSF-dependent cell line, TF-1/fms, which overexpresses functional Fms proteins on transfection of c-fms cDNA in TF-1, we obtained the same results as described above (Fig. 1B). The time courses of phosphorylation after M-CSF stimulation were similar between Cbl and Fms.

3.2. The phosphorylation of tyrosine 723 is important for the association of Cbl with Fms

For the analysis of the Cbl phosphorylation and signal transduction pathways of Cbl, we established a series of TF-1 sublines that expressed mutant Fms. The mutations were tyrosine (Y)-phenylalanine (F) substitutions of Y561, Y699, Y723 and Y809 in Fms.

Prior to analyzing the phosphorylation and association, we verified that all transfectants express the same amount of Fms proteins by flow cytometric analysis (data not shown) and immunoblotting (Fig. 2A). We also confirmed that the predicted associations of signaling molecules with Fms were abrogated by the introduction of mutation. In the clone F561, the M-CSF-dependent association between one of the Src

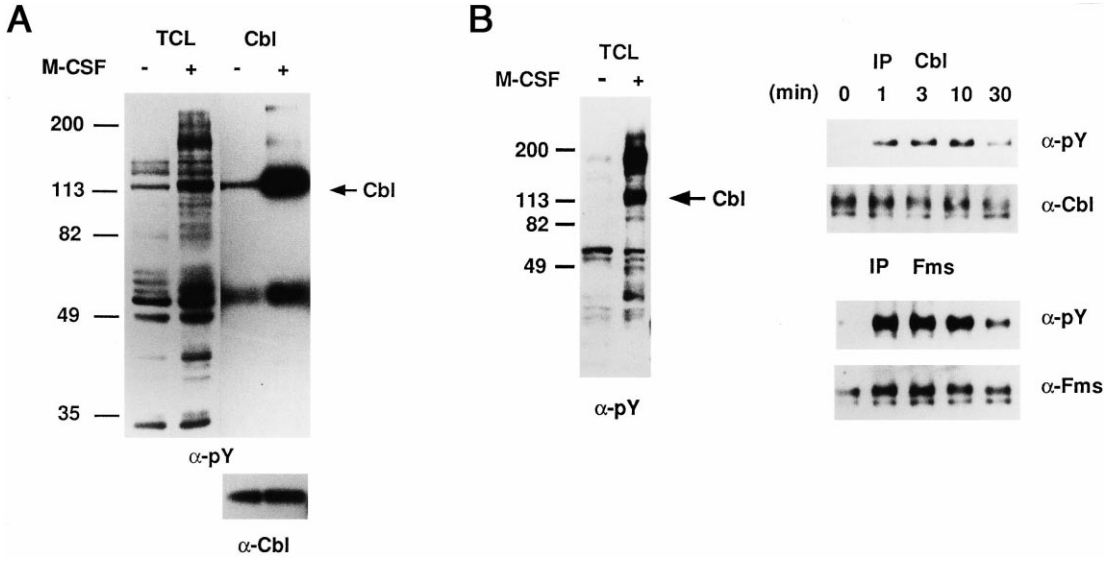

Fig. 1. Tyrosine phosphorylation of Cbl in bone marrow macrophages and TF-1/fms cells upon M-CSF stimulation. The total cell lysates and immunoprecipitates of anti-Cbl antibody of quiescent or M-CSF-stimulated bone marrow macrophages were separated by 8% SDS-PAGE under reducing conditions, then immunoblotted with anti-phosphotyrosine antibody. Molecular weight standards (pre-stained markers, Bio-Rad) are on the left (A). Results of the same experiments using TF-1/fms cells are shown in the left panel of B. TF-1/fms cells were lysed at the indicated times after M-CSF stimulation, immunoprecipitated with anti-Cbl or anti-Fms antibodies and immunoblotted with anti-phosphotyrosine, anti-Cbl, or anti-Fms antibodies (right panel in B).

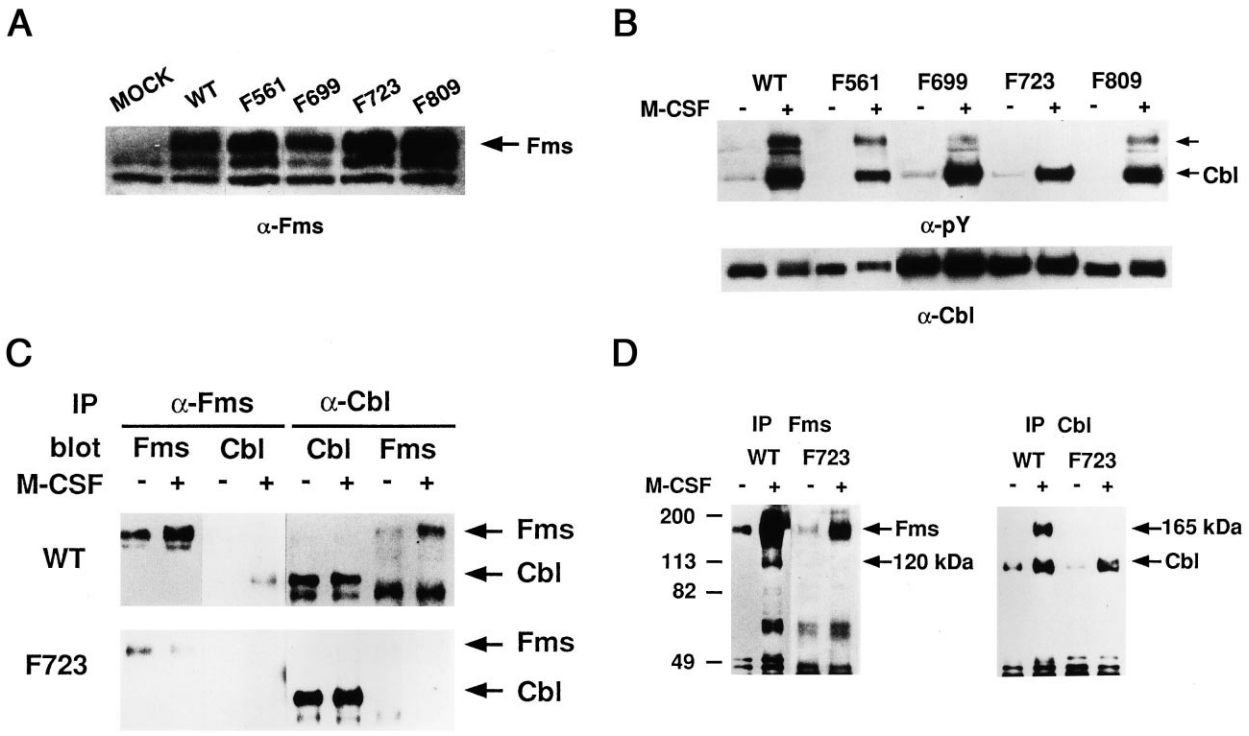

Fig. 2. Tyrosine phosphorylation of Cbl and its association with Fms in the wild-type (WT) or mutant c-fms-transfected TF-1 cells by M-CSF stimulation. Total cell lysates of MOCK, WT, and mutant Fms-expressing TF-1 cells were immunoblotted with anti-Fms antibody (A). Cells expressing WT and mutant Fms were either unstimulated or stimulated with M-CSF and their lysates were immunoprecipitated with anti-Cbl antibody. The precipitates were separated as described in Fig. 1A and immunoblotted with anti-phosphotyrosine or anti-Cbl antibodies. The arrowhead points to the protein with a molecular weight of about 150 kDa that was coprecipitated with Cbl (B). TF-1 cells expressing WT (upper panel) and F723 (lower panel) Fms were incubated in the absence or presence of M-CSF, then lysed and immunoprecipitated with anti-Fms or anti-Cbl antibodies. The precipitates were separated as described in Fig. 1A and immunoblotted with anti-Fms or anti-Cbl antibodies (C). The same samples as C were immunoblotted with anti-phosphotyrosine antibody. Molecular weight standards (pre-stained markers, Bio-Rad) are on the left (D).

family proteins, Lyn, and Fms was abrogated (data not shown). Unexpectedly, the association between Grb2 and Fms was not completely abrogated in clone F699 [20] (data not shown), unlike in a previous study using a fibroblast cell line [8]. In hematopoietic cell lines such as TF-1, it is difficult to obtain clear cut results of association particularly in the case of small adapter molecules like Grb2, because they indirectly associate with Fms via binding to other signaling molecules such as Shc, which is known to associate with Fms. In clone F723, the association between p85 and Fms was completely abrogated [7] (data not shown).

Cbl was immunoprecipitated by anti-Cbl antibody and immunoblotted with anti-phosphotyrosine or anti-Cbl antibodies. The phosphorylation of Cbl was induced in all mutant Fms-expressing clones as in wild-type clones (Fig. 2B). The relative levels of Cbl phosphorylation were 100, 54, 34, 28 and 55 in WT, F561, F699, F723 and F809 clones, respectively, when the intensities of phosphorylated Cbl were normalized with the amount of immunoprecipitated Cbl proteins. The differences in the quantities of Cbl protein in Fig. 2B were not due to a change in the expression levels of Cbl proteins on the transfection of various mutant c-fms constructs. Although the level of phosphorylation of Cbl was decreased in the mutant Fms-expressing clones, especially in F699 and F723, its phosphorylation was not completely abrogated by any of the mutations examined (Fig. 2B). We have confirmed these results by using three independent clones. Next, we compared the amount of phosphorylated protein with a molecular weight of about 150 kDa that was co-precipitated with Cbl (arrowhead in Fig. 2B). The 150 kDa protein signals did not appear in clone F723 (mutation in the p85 and PLC-γ2 binding sites) and appeared weakly in clone F699 (mutation in the Grb2 binding site) when compared with that of wild-type, F561 and F809 (Fig. 2B). As $p165^{Fms}$ is a tyrosine-phosphorylated protein, we investigated whether the 150 kDa Cbl-associated protein was Fms or not. As shown in Fig. 2C, Cbl was found in the anti-Fms immunocomplexes in WT cells and vice versa. However, no association between Cbl and Fms in F723 cells was observed (Fig. 2C, lower panel). These results show that Cbl associates with Fms by M-CSF stimulation, and phosphorylation of tyrosine 723 is particularly important for the association of Cbl with Fms. When the immunopresipitates of anti-Fms antibody were blotted with anti-phosphotyrosine antibody, a 120 kDa protein, which was likely a phosphorylated Cbl, was observed in the wild-type, but not detected in clone F723 (Fig. 2D, left panel). Conversely, a phosphorylated protein was present at the same position as $p165^{Fms}$ in immunoprecipitate of anti-Cbl antibody in the wild-type cells, but was absent in the F723 cells (Fig. 2D, right panel). The results also indicated that the tyrosine phosphorylation of Cbl occurred to a certain degree upon stimulation by M-CSF irrespective of an association with Fms.

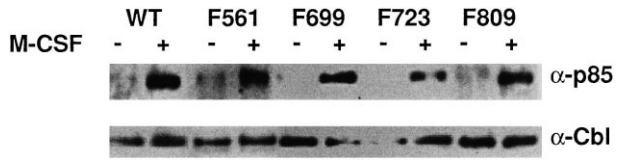

Fig. 3. M-CSF-dependent association of Cbl with p85 in wild-type (WT) or mutant c-fms-transfected TF-1 cells by M-CSF stimulation. The TF-1 cells expressing WT and mutant Fms were incubated in the absence or presence of M-CSF, then lysed and immunoprecipitated with anti-Cbl antibody. The precipitates were separated as described in Fig. 1A and immunoblotted with anti-p85 or anti-Cbl antibodies.

3.3. *M-CSF stimulation-dependent association of Cbl with the p85 subunit of PI3-kinase was not impaired by F723 and other mutations*

Recent findings revealed that membrane localization of PI3-kinase is necessary for alteration of the cytoskeleton in macrophages, and this requires the ligand stimulation-dependent association between Cbl and the p85 subunit of the PI3-kinase [33]. We therefore examined the association of Cbl with p85 upon M-CSF stimulation of TF-1/fms cells. Immunoprecipitation using anti-Cbl antibody followed by immunoblotting with the anti-p85 antibody revealed that the p85 subunit was co-precipitated with Cbl in an M-CSF-dependent manner in the wild-type and the mutant fms transfectants including cell line F723 (Fig. 3), in which Cbl was tyrosine-phosphorylated upon M-CSF stimulation but did not associate with Fms, as shown in Fig. 2B. These results indicate that M-CSF induces the association of Cbl with p85 in vivo, irrespective of the association of both p85 and Cbl with Fms.

4. Discussion

In this study, we analyzed the tyrosine phosphorylation of Cbl and association with Fms and other signal-transducing molecules upon M-CSF stimulation using various cell clones that express a series of tyrosine-phenylalanine-substituted Fms. In our system, the substitution of 723 resulted in marked impairment of the Cbl-Fms association (Fig. 2). However, it should be mentioned that Cbl was phosphorylated at its tyrosine, although to a lesser extent (Fig. 2B), and associated with p85 (Fig. 3) upon M-CSF stimulation in the mutant cells with Y723F substitution. The abrogation of the association between Cbl and Fms in clone F723 was not due to the decreased phosphorylation of Cbl, because the association was not detectable in F723 even when the same amount of phosphorylated Cbl compared to that in wild-type was precipitated (Fig. 2D, right panel). These results indicate that the phosphorylation of Cbl and its association with p85 do not completely depend on the association of the Cbl with activated Fms and suggest that a part of Cbl is phosphorylated by a tyrosine kinase other than Fms.

Tyrosine phosphorylation of Cbl is a common event in growth factor and cytokine signaling. Previous works have revealed that Src is responsible for Cbl phosphorylation in osteoclasts using src-deficient mice, and Cbl phosphorylation was impaired in B cells and T cells by targeting disruption of lyn and fyn, respectively [34,35]. In peritoneal macrophages, a triple mutation of $hck^{-/-}$, $fgr^{-/-}$ and $lyn^{-/-}$ but not a single mutation of each impaired the phosphorylation of Cbl in response to integrin-mediated adhesion [33]. These studies provide evidence that Src family kinases are responsible for Cbl phosphorylation in many systems. In M-CSF signaling, however, the mutation of Y561 (possible binding site for Src family kinases) did not impair the phosphorylation of Cbl (Fig. 2), suggesting that a kinase other than the Src family kinases played a role in this phosphorylation. We observed a difference in the phosphorylation pattern of cellular proteins between the wild-type and F561 mutant: this mutation abrogated tyrosine phosphorylation of STAT3, but not of Tyk2 (data not shown). Therefore, the phosphorylation of Cbl might be dependent on kinases activated by Fms such as Tyk2.

Tyrosine 723 was originally identified as the docking site for p85 [7,11]. Recently, it was indicated that PLC-γ2 also binds to the phosphorylated Y723 as p85 does [13]. PLC-γ2 is a 150 kDa protein with SH2 and SH3 domains that was reported to bind to Cbl via its SH2 domain in in vitro experiments using Jurkat cells [36]. In our in vivo immunoprecipitation experiments, M-CSF stimulation did not induce an association of Cbl with PLC-γ2 (data not shown), suggesting that the binding of Cbl to Y723 was not mediated by PLC-γ2. While p85 associates both with Fms and Cbl, it remains undefined whether Cbl associates indirectly with phosphorylated Y723 via binding to p85. Notably, two of the phosphorylation sites in Cbl contain the phosphotyrosine containing motif, pTyr-X-X-Met (pY371 and pY731), that is compatible with the binding preference of both N-terminal and C-terminal SH2 domains of p85 [37]. It should also be noted that there are two such motifs in activated M-CSF receptor dimer at pY723. It is not clear, however, whether two SH2 domains of p85 bind to the same molecule (Cbl or Fms), or two distinct molecules (Cbl and Fms). We observed that the association of Cbl with Fms was considerably impaired by the mutation of tyrosine 699 (Fig. 2), suggesting that Grb2 also participates in the Cbl-Fms interaction. However, this tyrosine seems to be less important for these interactions than tyrosine 723.

Cbl associates with some growth factor receptors, including EGF and PDGF receptors, directly via its PTB domain [38,39]. The predicted amino acid sequence of the PTB binding motif was Asp-Asp/Glu-X-pTyr [40]. Although there is no such consensus sequence in the cytoplasmic region of Fms, it is notable that the sequence surrounding Y723 is Val-Asp-Thr-Tyr. The Asp at the −2 position of Tyr was found to be important for recognition by the Cbl-PTB domain [40]. We therefore could not exclude the possibility that Cbl associates with Fms directly via its PTB domain.

One of the physiological functions of Cbl is thought to be the regulation of the cytoskeleton, which is required to exhibit differentiation specific functions such as bone remodeling of osteoclasts or migration of peritoneal macrophages [33,34]. Another function of Cbl, which is suspected in *Caenorhabditis elegans*, *Drosophila* and mammals, is negative regulation of receptor tyrosine kinase [41–43]. One mechanism in the negative regulatory function is demonstrated in mast cell and T cell signalings. In these cells, Cbl interacts with Syk and ZAP-70 in response to immunoglobulin receptor and T cell receptor engagement, respectively, and inhibits the activity of these tyrosine kinases [24,44]. However, the physiological functions of Cbl in the M-CSF signal cascade have not been elucidated.

The phosphorylation of Cbl is important for its association

with p85 (Fig. 3). Therefore, the identification of the tyrosine kinase(s) that physiologically phosphorylate(s) Cbl is required to understand the role of Cbl in M-CSF signaling.

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