6

Regulation of Tyrosine Kinase Signaling by Cbl in Hematopoietic Stem Cells

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1. Introduction

Phosphorylation of tyrosine residues is an essential biochemical reaction in many higher eukaryotes. One of the most important and well-studied functions of tyrosine phosphorylation is to convey extracellular signals to the cytoplasm and ultimately to the nucleus in order to control various cell functions such as proliferation, differentiation, migration and survival.

The first tyrosine kinase (TK) was discovered as the tumor-inducing activity from Rous sarcoma virus, which is now known as v-Src (Rous, 1911). Later studies revealed that the oncogenic properties of v-Src was due to the loss of the regulatory mechanisms to control its kinase activity (Martin, 2001). These findings clearly highlight the critical importance of precise regulation of TK activities in order to avoid detrimental consequences to the homeostasis of the organisms.

Various mechanisms are employed to control TK activities. In the Src family non-receptor tyrosine kinase (non-RTK), phosphorylation status of the tyrosine residue in the C-terminal regulatory region alters intramolecular interactions and therefore serves as a way to modulate kinase activity. The kinases and the phosphatases involved in this regulatory mechanism are, in turn, themselves under additional layers of regulation, thus, creating an intricate network of signal mediators to fine-tune cellular responses (Sen & Johnson, 2011). Incidentally, activity of a typical receptor tyrosine kinase (RTK) is regulated by ligand binding; RTKs alter conformation upon ligand binding and dimerize, which leads to transphosphorylation of critical tyrosine residues in the activation loop of the neighboring kinase in the cytoplasmic domain (He & Hristova). This initiates a cascade of biochemical reactions that activates downstream signaling pathways.

Subcellular localization of TKs is another important determinant of their activity (Murphy et al., 2009). There are now abundant evidence indicating that TKs can generate different signals dependent on their intracellular locations. Therefore, molecules that regulate protein trafficking and localization constitute a critical component of signal regulatory mechanism.

Covalent attachment of small proteins such as ubiquitin and small ubiquitin-like modifier (SUMO) to target proteins serves as a signal for various biological processing, including

alteration of its localization and promotion of degradation (Schulman & Harper, 2009; van Wijk & Timmers, 2010). This reaction is mediated by a series of biochemical reactions involving the E1 or activating enzyme, the E2 or conjugating enzyme and the E3 ligase. Human genome encodes for two E1s, thirty E2 and over one thousand E3s for the ubiquitin system. This pathway architecture immediately implies that the substrate specificity of the ubiquitin system must be achieved largely at the level of E3s.

The Casitas B-lineage lymphoma (Cbl) family proteins are RING finger (RF)-containing multi-domain adaptors that function as E3 ubiquitin ligase primarily towards activated TKs (Thien & Langdon, 2001; Duan et al., 2004; Schmidt & Dikic, 2005). Using genetically-engineered mouse models, we and others showed that loss of Cbl, either singly or in combination with another family member Cbl-b, led to the enlargement of the hematopoietic stem cell (HSC) compartment (Naramura et al., 2011a). Additionally, mutations in the *CBL* gene have been identified in a small but significant number of hematological malignancies in human, and experimental evidence proved the oncogenicity of mutant *CBL* products (Naramura et al., 2011b). All together, these observations strongly support that the Cbl family proteins are critical regulators of hematopoietic homeostasis.

Here, we review functions of the Cbl family proteins and some of the candidate Cbl targets in the HSC compartment and discuss potential mechanisms of their regulation.

2. The Cbl family proteins

The Cbl family proteins are evolutionarily conserved signal regulators present through *C. elegans* to human (Figure 1). In mammals, this family includes Cbl (also known as c-Cbl, encoded by the *CBL* gene in human), Cbl-b (*CBLB* gene in human) and Cbl-c (also know as Cbl-3 or Cbl-SL, *CBLC* gene in human). Cbl was originally identified as a cellular homolog of a viral oncogene *v-Cbl* which caused leukemia and lymphoma in mice (Langdon et al., 1989). Cbl's involvement in signal transduction was suggested because it became prominently tyrosine-phosphorylated upon stimulation through various cell surface receptors (Donovan et al., 1994; Galisteo et al., 1995). But it was not until genetic studies in *C. elegans* identified the *sli-1* gene product as a Cbl homolog that Cbl was established as a negative regulator of RTK signaling.

2.1 Structure and biochemical functions of the Cbl family proteins

All Cbl family proteins share a high degree of homology in their N-terminal regions. These include the tyrosine kinase binding (TKB) domain, the RF domain and the short intervening linker region. X-ray crystallography studies revealed that the TKB domain comprised a four-helix bundle (4H), a calcium-binding EF hand and a variant Src homology region 2 (SH2) domain (Meng et al., 1999). The TKB domain mediates specific binding to cognate phosphotyrosine-containing motifs in activated TKs and select non-TK signal mediators (Lupher et al., 1996). The RF domain and the linker region together bind to E2 ubiquitin-conjugating enzymes and both of these motifs are essential for the E3 ubiquitin ligase activity of the Cbl family proteins (Joazeiro et al., 1999; Levkowitz et al., 1999; Yokouchi et al., 1999; Zheng et al., 2000).

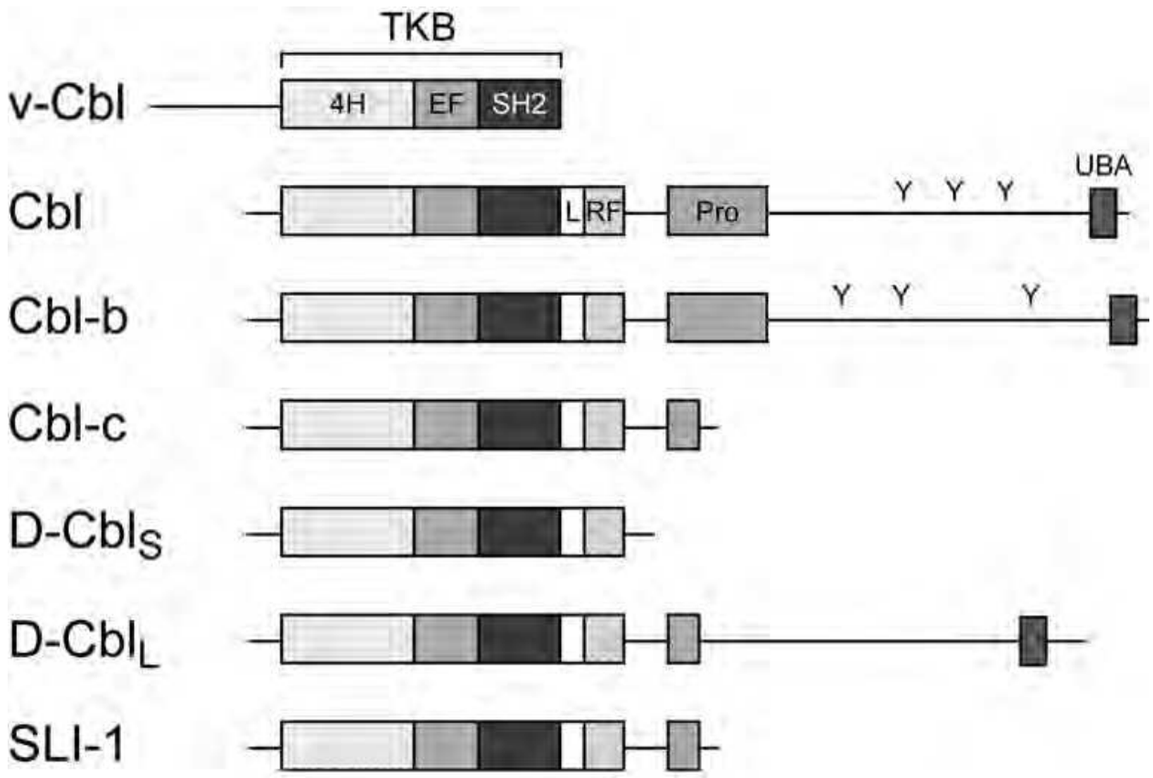

Fig. 1. Structure of the Cbl family proteins. The original oncogenic form of Cbl (v-Cbl), the three mammalian Cbl family proteins (Cbl, Cbl-b and Cbl-c), the short and long forms of *Drosophila* Cbl (D-Cbl$_S$ and D-Cbl$_L$) and the *C. elegans* homolog (SLI-1) are shown. TKB, tyrosine kinase binding; 4H, four-helix bundle; EF, EF hand; SH2, Src homology region 2; L, linker; RF, RING finger; Y, tyrosine; UBA, ubiquitin-associated.

Band and colleagues originally described that the Cbl TKB domain specifically recognized the phosphotyrosine-containing motif D(N/D)XpY, which was later refined as (N/D)XpY(S/T)XXP, found in several TKs such as ZAP70, epidermal growth factor receptor (EGFR), and Src (Lupher et al., 1997). Additional binding motifs, RA(V/I)XNQpY(S/T) and DpYR, were proposed in the adaptor protein APS (Hu & Hubbard, 2005) and the RTK c-Met (also known as hepatocyte growth factor receptor; Peschard et al., 2004), respectively. A recent comprehensive structural study showed that phosphopeptides with diverse sequences bound TKB at the same site, albeit in two different orientations (Ng et al., 2008). These studies collectively revealed the unique binding strategy for the specialized and biologically vital function of the Cbl family proteins and provided means to identify potential Cbl targets based on the amino acid sequences.

The C-terminal half of the Cbl family proteins are more divergent. A proline-rich region follows the RF domain in all mammalian Cbl family proteins, but this domain is more prominent in Cbl and Cbl-b than in Cbl-c. Biochemical studies have demonstrated that Cbl interacted with SH3-domain containing proteins such as Grb2 and Nck through the proline-rich region (Rivero-Lezcano et al., 1994; Fukazawa et al., 1995).

In addition to being a TK regulator, Cbl itself is subject to tyrosine phosphorylation. Phosphorylation at tyrosine residues 700, 731 and 774 have been extensively characterized; residues 700 and 774 provide docking sites for the SH2 domain-containing adaptor protein CrkL (Andoniou et al., 1996). Tyrosine 700 also mediates an interaction with the guanine nucleotide exchange factor Vav (Marengère et al., 1997). Tyrosine 731 provides a docking site of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Hunter et al., 1999). Based on sequence homology and experimental data, tyrosine residues in the C-terminal domain of Cbl-b are thought to share many of the same functions as those in Cbl. Cbl-c does not possess comparable tyrosines.

The C-termini of Cbl and Cbl-b, but not Cbl-c, contain a conserved domain known as a ubiquitin-associated (UBA) domain, which is present in a variety of proteins involved in ubiquitin-mediated processes. Structural studies indicate that this domain is capable of binding ubiquitin and involved in dimerization (Kozlov et al., 2007; Peschard et al., 2007).

2.2 Insights from genetic models

The first critical clue into Cbl's functions came from genetic studies in *C. elegans* (Yoon et al., 1995). The vulval development in *C. elegans* is regulated by signals through the EGFR pathway. A reduction-of-function mutation in *let-23* (encodes the EGFR homolog) leads to death of most worms, and the vulval development is incomplete in surviving worms. However, when loss-of-function mutations in *sli-1* were introduced to this genetic background, worms survived and vulval development was restored. The sequence analysis of the *sli-1* gene revealed that it encoded a protein with a high similarity to Cbl, thus establishing Cbl as a negative regulator of the EGFR pathway.

Genetic studies in gene targeted mouse models provided further insights into the physiological roles of the Cbl family proteins in mammals. Cbl-deficient mice are viable, but they show recognizable changes in the hematopoietic, lymphoid, metabolic and reproductive systems. In contrast, effects of Cbl-b loss is mostly limited to the peripheral immune functions. Cbl-c expression appears to be restricted to the epithelial tissues, but no significant phenotypes were reported in mice deficient in Cbl-c (Table 1).

While mice deficient in either one of the Cbl family members are viable, simultaneous loss of Cbl and Cbl-b is not compatible with the survival of the organism and double-deficient mice do not survive beyond embryonic day 10 (Naramura et al., 2002). This indicates that Cbl and Cbl-b play redundant and overlapping functions in critical organ systems during fetal development. Using the Cre-loxP-mediated conditional gene deletion approach, effects of Cbl, Cbl-b loss have been analyzed in the T, B and HSC compartments (Naramura et al., 2002; Huang et al., 2006; Kitaura et al., 2007; Naramura et al., 2010). These studies demonstrated that, in the adaptive immune system, the Cbl family proteins are required to establish appropriate threshold for selection of T and B cells, and disruption of this process leads to autoimmune-like phenotypes in mice.

In the hematopoietic compartment, Cbl-deficiency leads to moderate splenomegaly and enhanced extramedullary hematopoiesis (Murphy et al., 1998). In the bone marrow, the lineage-negative, Sca-1-positive, c-Kit-positive (LSK) compartment, which is highly enriched for HSCs, is enlarged and Cbl-deficient HSCs showed enhanced capacity to reconstitute myeloabrated recipient's hematopoietic system (Rathinam et al., 2008). However, mice were

outwardly normal and had a normal lifespan. When both Cbl and Cbl-b are deleted in the HSC, however, mice succumbed to aggressive myeloproliferative disease-like leukemia within two to three months after birth (Naramura et al., 2010).

Gene	Phenotype	Reference
Cbl	Altered T cell antigen receptor expression Increased tyrosine phosphorylation Enhanced thymic selection Splenomegaly and extramedullary hematopoiesis Decreased fertility Altered metabolism	(Murphy et al., 1998; Naramura et al., 1998; Thien et al., 1999; Molero et al., 2004; El Chami et al., 2005; Rathinam et al., 2008)
Cblb	Co-stiumlation-independent activation of peripheral T cells Predisposition to autoimmune diseases and inflammatory injury Resistance to spontaneous and transplanted tumors	(Bachmaier et al., 2000; Chiang et al., 2000; Krawczyk et al., 2000; Chiang et al., 2007; Loeser et al., 2007; Bachmaier et al., 2007)
Cblc	No apparent phenotypes	(Griffiths et al., 2003)

Table 1. Phenotypes of mice deficient in the Cbl family members

2.3 Cbl and hematological malignancies

Because of the involvement of various RTKs in cancer, it has long been speculated that the Cbl family proteins may play critical roles in the initiation and/or progression of cancer. Oncogenic mutations in RTKs that abrogate interaction with Cbl have been reported (Peschard & Park, 2003), but the direct evidence supporting Cbl's roles in cancer was not established until 2007.

The vast majority of *CBL* mutations reported so far are associated with myeloid disorders. Although the first human *CBL* mutations were described in acute myeloid leukemia (AML) samples (Sargin et al., 2007; Caligiuri et al., 2007; Abbas et al., 2008), later studies documented a significant number of cases in myelodysplastic syndromes-myeloproliferative neoplasms (MDS/MPN), a heterogeneous group of myeloid disorders including the chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML) and juvenile myelomonocytic leukemia (JMML) (Dunbar et al., 2008; Reindl et al., 2009; Grand et al., 2009; Loh et al., 2009; Sanada et al., 2009; Makishima et al., 2009; Muramatsu et al., 2010; Fernandes et al., 2010; Niemeyer et al., 2010). The association of *CBL* mutations with JMML is particularly thought-provoking because the pathogenesis of this rare pediatric hematological malignancy is closely associated with the activation of the Ras-MAPK signaling pathway (Loh, 2011). Among JMML patients, the activating mutations of *PTPN11*, *NRAS* and *KRAS*, and the loss of *NF1*, a gene encoding for a Ras GTPase-activator account for approximately 75 % of the total cases. Roughly half of the remainder of the cases are now attributed to *CBL* mutations. While *in vitro* experimental data indicate that the loss of the Cbl family proteins lead to prolonged Erk activation, it was never formally demonstrated whether Cbl can regulate Ras activity directly.

It is of note that most *CBL* mutations are either point mutation or internal deletion involving the linker and/or the RF regions rather than complete deletion at the *CBL* locus. As expected from domain-function analysis results, these mutant Cbl proteins lack E3 ubiquitin ligase activity. Interestingly, in patient samples with *CBL* mutations, the wild-type allele is often lost and replaced with the mutant allele by acquired uniparental isodisomy (aUPD). The *CBLB*, *CBLC* alleles are usually unaffected in these patients although mutations in these genes have been reported (Makishima et al., 2009; Makishima et al., 2011). All together, these clinical observations suggest that the presence of one wild-type copy of *CBL* is generally sufficient to maintain the functions of Cbl in the presence of normal Cbl-b and Cbl-c. These findings are consistent with the data in mice expressing a RF-mutant Cbl from the endogenous promoter on a *Cblb*, *Cblc* wild-type background (Thien et al., 2005; Rathinam et al., 2010); homozygous mutant mice are perinatally lethal, but hemizygous mutants over the wild-type *Cbl* allele develop normally. However, when the hemizygous mutant is expressed over the Cbl-null background, mice develop myeloproliferative disease-like leukemia within a year. This is a striking contrast when compared to the rapid progression and fatality of the HSC-specific Cbl, Cbl-b double-deficient mice (Naramura et al., 2010). These differences may reflect that the RF mutant and patient-derived oncogenic mutant Cbl proteins function as gain-of-function mutants rather than as dominant-negative inhibitors of Cbl-b (Cbl-c expression is minimal in the hematopoietic system). While these mutants lack E3 ubiquitin ligase activity and thus defective in promoting target degradation, they possess intact TKB and C-terminal protein-protein interaction motifs, which may enable them to form aberrant but stable multi-protein super-signaling complexes and activate unconventional signaling pathways.

2.4 Potential Cbl targets in the hematopoietic system

What, then, are the target of Cbl-dependent regulation in the HSC compartment? Because Cbl becomes phosphorylated upon stimulation with various cell surface receptors, it is conceivable that Cbl is involved in the regulation of signal transduction downstream of such pathways (Table 2). Among this diverse group of cell surface receptors, Kit and Flt3 are of particular interest because both of them are RTKs expressed in HSC and known to perform critical functions in the HSC compartment (Masson & Rönnstrand, 2009). Colony stimulating factor 1 receptor (CSF1R) is known to interact with Cbl (Lee et al., 1999), but it is expressed primarily in more differentiated myeloid/phagocytic cells than in HSCs. Endothelial-specific receptor tyrosine kinase (Tek, also known as Tie2) is another RTK expressed in the HSC compartment (Arai et al., 2004) and therefore may interact with Cbl. Thrombopoietin (TPO) is indispensable for the maintenance of HSC quiescence (Yoshihara et al., 2007; Qian et al., 2007). Although its receptor (TPO-R, also known as Mpl or c-Mpl) is not an RTK, stimulation with TPO induce phosphorylation of Cbl (Sasaki et al., 1995), Mpl have been shown to be ubiquitinylated (Saur et al., 2010) and Cbl loss alters the signal transduction downstream of TPO (Rathinam et al., 2008; Naramura et al., 2010). Therefore, Mpl may interact with Cbl indirectly. Other potential (direct as well as indirect) Cbl targets in the HSC compartment include the chemokine and integrin pathways.

In following sections, I will discuss how these pathways may be regulated by the Cbl family proteins in the HSCs.

Antigen and other immunological receptors • T cell antigen receptor complex (Donovan et al., 1994; Meisner et al., 1995; Fukazawa et al., 1995) • B cell antigen receptor complex (Cory et al., 1995; Tezuka et al., 1996; Panchamoorthy et al., 1996) • Fcγ receptor (Marcilla et al., 1995) • Fcε receptor (Matsuo et al., 1996; Suzuki et al., 1997)
RTKs • Epidermal growth factor receptor (Galisteo et al., 1995) • Insulin receptor (Ribon & Saltiel, 1997) • Platelet-derived growth factor receptor (Bonita et al., 1997) • Kit (Wisniewski et al., 1996; Brizzi et al., 1996) • Flt3 (Lavagna-Sévenier et al., 1998) • Fibroblast growth factor receptor (Wong et al., 2002) • Colony stimulating factor 1 receptor (Wang et al., 1996) • Met (Fixman et al., 1997; Garcia-Guzman et al., 2000) • TrkB (McCarty & Feinstein, 1999) • Tie2 (Wehrle et al., 2009)
Cytokine receptors • Interleukin 2 receptor (Gesbert et al., 1998) • Interleukin 3 receptor (Barber et al., 1997) • Interleukin 4 receptor (Ueno et al., 1998) • Erythropoietin receptor (Odai et al., 1995; Barber et al., 1997) • Mpl (Sasaki et al., 1995; Brizzi et al., 1996) • GM-CSF receptor (Odai et al., 1995) • Prolactin receptor (Hunter et al., 1997)
Chemokine receptors (Chernock et al., 2001)
Integrins (Ojaniemi et al., 1997; Manié et al., 1997; Meng & Lowell, 1998)

Table 2. Partial list of potential upstream receptors for Cbl

3. Kit

The mouse dominant spotting mutation at the *W* locus was first described in the early 1900s (Durham, 1908). Mutations at this locus were studied extensively not only because they produced visible coat color changes, but also because mutant mice showed defects in hematopoiesis, mast cell development and gametogenesis (Russell, 1979). However, it was not until 1988 that the gene product at the *W* locus was found to encode for the cellular homolog of the *kit* oncogene which had been molecularly identified a few years earlier (Besmer et al., 1986; Chabot et al., 1988; Geissler et al., 1988).

Kit is a type III RTK that shares structural similarities with platelet-derived growth factor receptors (PDGFRs) α and β, Flt3 (also known as Flk-2, discussed below) and CSF1R. They

are characterized by an extracellular domain with five immunoglobulin-like domains, a single transmembrane domain and an intracellular tyrosine kinase domain that is split into two by an intervening sequence.

HSCs are functionally defined as rare cells with the capacity to self-renew and give rise to all cell types of the hematopoietic lineage, including erythrocytes, granulocytes, monocytes, megakaryocytes and lymphocytes. No single marker specific for HSCs is known today. However, it is widely accepted that, in mice, most HSCs reside in a population of cells that express Kit and another cell surface protein Sca-1 and lack the expression of committed lineage markers (Ikuta & Weissman, 1992). Thus, Kit expression is intimately tied to HSCs.

The ligand for Kit is called stem cell factor (SCF) and encoded by the Steel (*Sl*) locus. The phenotypes of the *Sl* mutant mice are in most cases similar to those of *W* mutant mice, affecting hematopoiesis, mast cell development, fertility and coat colors (Galli et al., 1993). Collectively, these observations firmly established the essential roles of the SCF-Kit axis in these biological processes.

In the mouse embryo, hematopoietic cells are found in the blood islands in the yolk sac starting around embryonic day 7. Subsequently, at day 10-11 of gestation, HSCs migrate to the fetal liver and then to the spleen and the bone marrow, the primary hematopoietic organs in adult. The hematopoietic defect in *W* mice is detected throughout the course of development. Syngeneic transplantation experiments demonstrated that the defect exerted by *W* mutations was intrinsic to hematopoietic cells. The hematopoietic microenvironment in these animals are not affected and able to support hematopoiesis of normal donor-derived cells (Russell, 1979).

Kit activity is regulated at various levels. Ligand-receptor engagement of Kit initiates receptor dimerization and subsequent activation of its TK activity. Extensive biochemical studies have mapped intracellular phosphorylated tyrosine residues and their interacting proteins. These include Src family TKs, phosphatases such as SHP1 and SHP2, phospholipase Cγ, p85 subunit of phosphoinositide-3 kinase, (p85(PI3K)) and adaptor proteins such as Grb2 and APS (Lennartsson et al., 2005).

Cbl becomes phosphorylated when Kit-expressing cells are stimulated with SCF (Wisniewski et al., 1996). Earlier studies suggested that Cbl interacted with Kit indirectly through Grb2 (Brizzi et al., 1996), CrkL and p85(PI3K) (Sattler et al., 1997), and APS (Wollberg et al., 2003). More recent data suggest that Cbl binds to Kit directly at tyrosine 568, which is located in the juxtamembrane domain, and tyrosine 936, which is located in the carboxyterminal tail, ubiquitinylate Kit and target them for degradation (Masson et al., 2006). Both Cbl and Cbl-b function similarly towards Kit (Zeng et al., 2005). Hematopoietic cells deficient in Cbl functions are hypersensitive to stimulations through Kit (Naramura et al., 2010; Rathinam et al., 2010). These data all together strongly support that Kit may be one of the physiological targets of Cbl proteins in the HSC compartment.

Structurally, sequences surrounding tyrosine 568 partially conform to the canonical Cbl(TKB) recognition sequence while those around tyrosine 936 do not. Further analyses into the mechanisms of binding between Cbl and Kit may reveal novel molecular interactions that remained unknown so far.

4. Flt3

Flt3, another member of the type III RTKs, was originally identified by two separate groups through homology screening for TKs (Matthews et al., 1991; Rosnet et al., 1991). Its expression is detected in placenta, gonads, brain and hematopoietic cells, but its role outside of the hematopoietic system is not clear at present. Ligand for Flt3 (Flt3 ligand; FL) was identified a few years later and its transcript is expressed in wide range of both fetal and adult tissues (Lyman et al., 1993; Hannum et al., 1994).

Roles of Flt3 in HSCs appear to vary among species and also dependent upon developmental stages. The most primitive self-renewing HSCs with long-term reconstituting potential (LT-HSCs) are not found within $Flt3^+$ LSK cells in adult mouse bone marrow while the same biological activity was detected in both $Flt3^+$ and $Flt3^-$ populations in fetal liver (Adolfsson et al., 2001; Christensen & Weissman, 2001). Notably, human HSCs with multi-lineage reconstituting activity are $Flt3^+$ (Sitnicka et al., 2003). Flt3 deficient mice are viable and fertile, but show defects in B lymphocyte progenitors and dendritic cell generation (Mackarehtschian et al., 1995). The role of the FL-Flt3 axis in HSC maintenance and expansion remains controversial. Mackarehtschian et al. originally reported that Flt3-deficient bone marrow cells showed defects in lymphoid and myeloid reconstitution upon transplanting into myeloablated hosts (Mackarehtschian et al., 1995), while a more recent report by Buza-Vidas et al. concluded that Flt3 and FL were dispensable for maintenance and posttransplantation expansion of mouse HSCs (Buza-Vidas et al., 2009). Partly based on the phenotypes of Flt3 and FL deficient mice, models were proposed that Flt3 might function in the lineage restriction process from HSCs to lymphoid progenitors (Luc et al., 2007). However, in human, activating mutations in the *FLT3* gene, either in the form of internal tandem duplication (ITD) mutation in the juxtamembrane domain or point mutations in the kinase domain, are more frequently associated with myeloid malignancies rather than with lymphoid malignancies (Stirewalt & Radich, 2003). Clearly, the roles of Flt3 in the normal and pathological hematopoiesis need to be further delineated.

Cbl becomes tyrosine phosphorylated upon Flt3 engagement (Lavagna-Sévenier et al., 1998). It has been shown to physically interact with Flt3, and overexpression of an E3 ligase-defective mutant Cbl inhibited FL-induced Flt3 ubiquitylation and internalization, indicating involvement of Cbl in Flt3 signaling regulation (Sargin et al., 2007). Mice expressing a RF mutant Cbl from its endogenous locus are hypersensitive to FL stimulation (Rathinam et al., 2010), and we confirmed a similar phenotype in mouse bone marrow cells deficient in both Cbl and Cbl-b (Naramura, manuscript in preparation). Furthermore, deletion of FL blocks leukemia development in Cbl RING finger mutant mice (Rathinam et al., 2010).

Nevertheless, the mode of interaction between Cbl and Flt3 has not been clarified. Direct binding between Cbl and Flt3 has not been demonstrated. The sequences surrounding tyrosine 589 partially conform to the canonical Cbl(TKB) recognition sequence, and this region shares a very high homology to the sequences surrounding tyrosine 568 (a candidate Cbl binding site) in Kit. Notably, this is also the region frequently affected by ITD mutations. Alternatively, or in addition to the direct binding, because Flt3 is known to interact with Grb2 (Dosil et al., 1993; Zhang et al., 1999), a Cbl-binding adaptor protein, Cbl-Flt3 interaction may be mediated through this adaptor protein.

5. Other potential targets

As is clear from the list of potential Cbl upstream receptors, Kit and Flt3 may not be the only targets of Cbl-dependent regulation in HSCs. Although pathways other than Kit or Flt3 have not been as carefully examined in relation to Cbl, existing evidences suggest that following pathways may be regulated by the Cbl family proteins either directly or indirectly.

5.1 Tek

Tie2, encoded by the *TEK* gene, is an RTK expressed predominantly on endothelial cells, but they also provide crucial functions in the maintenance of quiescence and self-renewal capacity of the HSCs (Arai et al., 2004). The interaction between Tie2 and angiopoietin-1 (Ang-1), its ligand, has been shown to promote ubiquinylation of Tie2 by Cbl and receptor internalization (Wehrle et al., 2009). Structurally, the cytoplasmic domain of Tie2 does not contain any tyrosine residues that match the canonical Cbl(TKB) recognition sequence. However, activated Tie2 is known to bind Grb2 (Huang et al., 1995), thus may interact with Cbl indirectly through this adaptor.

5.2 Cytokine receptors

Cytokines such as hematopoietic growth factors and interleukins play essential roles in hematopoiesis. Cbl becomes tyrosine phosphorylated upon stimulation through various cytokine receptors (Table 2), and hematopoietic cells deficient in Cbl activity show enhanced sensitivity to cytokines (Rathinam et al., 2008; Sanada et al., 2009; Naramura et al., 2010). Receptors for these factors do not possess cytoplasmic tyrosine kinases but they activate the Janus kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway (Yoshimura, 2009). Ligand binding induces receptor oligomerization, which activate associated JAK kinases and they, in turn, phosphorylate the receptor cytoplasmic domains and create binding sites for SH2-containing proteins.

There is no solid experimental evidence supporting the direct interaction between the JAK/STAT pathway and Cbl. Activation of the JAK/STAT pathway induces the expression of Suppressor of Cytokine Signaling (SOCS) family proteins, which function as E3 ubiquitin ligases for this pathway.

In addition to the JAK/STAT pathway, ligand binding to cytokine receptors activate the Ras-MAPK pathway through adaptor proteins such as APS and Grb2. Activation of this pathway is required for cell proliferation. As discussed above, these adaptor proteins are know to interact with Cbl, providing a potential link between the cytokine pathway and the Cbl family proteins.

6. Conclusion

In spite of its original identification as a cellular homolog of a viral oncogene, pathophysiological roles of the Cbl family proteins remained unclear for some time. Genetic studies in model organisms as well as identification of *CBL* mutations in patient-derived specimen played crucial roles in deciphering their essential functions as regulators of HSC homeostasis. Combined with molecular/biochemical information gathered over the last two decades, we now appreciate the complexity of the regulatory pathways surrounding the Cbl

family proteins. While the primary focus of studies in the last ten years has been on Cbl's E3 ubiquitin ligase functions towards phosphotyrosine motif-containing targets, observations in cells expressing mutant Cbl proteins began to challenge this relatively-simplistic viewpoint. Further studies into this multifaceted protein family may uncover opportunities for novel diagnostics and therapeutics.

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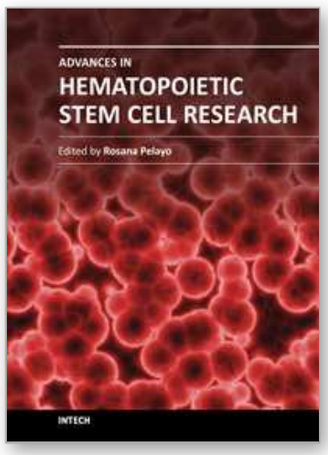

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This book provides a comprehensive overview in our understanding of the biology and therapeutic potential of hematopoietic stem cells, and is aimed at those engaged in stem cell research: undergraduate and postgraduate science students, investigators and clinicians. Starting from fundamental principles in hematopoiesis, Advances in Hematopoietic Stem Cell Research assemble a wealth of information relevant to central mechanisms that may regulate differentiation, and expansion of hematopoietic stem cells in normal conditions and during disease.

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