

Cbl Family Proteins: Balancing FcεRI-Mediated Mast Cell and Basophil Activation

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Mast cells • Basophils • FcεRI • Negative regulation •
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Abstract

The binding of IgE to high-affinity IgE receptors (FcεRI) expressed on the surface of mast cells and basophils initiates a cascade of signaling events that results in the release of a wide array of proinflammatory mediators. In order to limit the intensity and duration of cell activation, FcεRI aggregation has been understood to additionally generate negative signals through the coordinated action of adapters, phosphatases, and ubiquitin ligases. Among them, Cbl family proteins negatively regulate FcεRI-mediated signals mainly by promoting ubiquitination of the activated receptor subunits and associated protein tyrosine kinases. Notably, FcεRI ubiquitination has become recognized as an important signal for the internalization and delivery of engaged receptor complexes to lysosomes for degradation. The surface expression of activated FcεRI complexes is further downregulated through a pathway that is functionally separable from Cbl ligase activity and is dependent on the interaction of Cbl proteins with adapters involved in clathrin-dependent endocytosis. In this article, we review recent advances in our understanding of the molecular mechanisms through which Cbl proteins negatively regulate FcεRI-mediated mast cell and basophil functions.

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Introduction

Mast cells and basophils share the surface expression of the high-affinity receptor for IgE (FcεRI), which upon engagement transduces intracellular signaling, leading to the release of granule-stored and newly synthesized proinflammatory mediators [1–4].

Besides these positive signals, FcεRI aggregation has been understood to generate negative intracellular signals capable of limiting basophil and mast cell functional responses through the action of a variety of multidomain adapter proteins [5].

Among them, the Cbl family of ubiquitin (Ub) ligases [6–8] has attracted considerable interest due to the finding that it controls the intensity and duration of FcεRI-mediated signals mainly by the specific Ub modification of the activated receptor subunits and associated protein tyrosine kinases (PTKs) [9, 10].

Cbl can also promote the internalization of engaged FcεRI through a pathway that is functionally separable from its Ub ligase activity and is dependent on Cbl interaction with a multidomain protein mainly involved in the process of clathrin-dependent endocytosis, i.e. Cbl-interacting protein of 85 kDa (CIN85) [11].

In this review we will discuss our current understanding of how Cbl proteins negatively regulate mast cell and basophil functional responses upon FcεRI stimulation.

Signaling through FcεRI

FcεRI is a multisubunit complex consisting of an IgE-binding α-chain, a 4-transmembrane-spanning β-chain with cytoplasmic N-terminal and C-terminal edges, and a disulphide-linked γ-γ homodimer [3, 12].

In rodents, all 3 subunits are needed for surface expression, whereas in humans a trimeric complex lacking the β-chain (FcεRIαγ₂) also exists and is expressed not only on mast cells and basophils but also on antigen-presenting cells [3].

The FcεRIβ subunit acts as an amplifier of FcεRI surface expression by controlling the proper trafficking and maturation of the α-chain [13].

FcεRI surface expression is strongly upregulated upon interaction with monomeric IgE in both basophils and mast cells [14–17]. Notably, the level of circulating IgE correlates with the cell surface density of FcεRI on human basophils [18–20]. Conversely, the treatment of atopic patients with anti-IgE antibodies decreases serum IgE levels and FcεRI surface expression [21].

Several observations, obtained using the rat basophilic leukemia cell line RBL-2H3, have contributed to elucidating the function of the different receptor subunits.

The FcεRI α-chain contains 2 extracellular Ig-like domains involved in IgE binding, a transmembrane region with an aspartic acid residue, and a short cytoplasmic tail that lacks signal transduction motifs; this chain has the exclusive role of recognizing the Fc portion of IgE.

The β and γ subunits share a conserved immunoreceptor tyrosine-based activation motif (ITAM) within their long cytoplasmic tails. Upon FcεRI aggregation, ITAMs are rapidly phosphorylated on tyrosines by the Src family kinase Lyn, which is constitutively and weakly associated with the FcεRI β subunit [2, 3].

The phosphorylated γ-chains are competent to drive cell activation in the absence of a β-chain ITAM; however, FcεRIβ amplifies the intensity of signals from FcεRIγ [22]. Thus, the 2 subunits act cooperatively in promoting signal transduction.

The full activation of FcεRI requires the recruitment of aggregated receptors into lipid rafts, i.e. membrane domains rich in cholesterol and sphingolipids that form ordered but dynamic structures floating in the less ordered surrounding membrane [23].

Upon FcεRI engagement, membrane rafts coalesce into larger and more stable structures where engaged receptors are concentrated [24] and can more easily interact

with signaling molecules, such as active Lyn [25, 26], favoring phosphorylation events.

Receptor phosphorylation is necessary for the activation of the cytoplasmic kinase Syk, which is recruited at the plasma membrane upon recognition of FcεRI phosphorylated ITAMs [2].

The use of specific Syk inhibitors and Syk-negative cell lines has demonstrated an obligatory role for Syk in FcεRI-mediated signaling [27–30]. For example, bone marrow-derived mast cells (BMMCs) isolated from Syk^{−/−} mice show impaired calcium mobilization, degranulation, and cytokine production upon receptor stimulation [28]. In humans, a minority of normal blood donors have basophils that fail to release granules. These ‘nonreleaser’ basophils express a normal level of FcεRI but very low levels of Syk protein [31, 32]. Furthermore, variable expression levels of Syk, observed in human basophils from releaser donors, correlate well with the IgE-mediated responsiveness of these cells [32].

The activation of Syk enables a productive interaction with many targets, including the membrane-anchored linker for activation of T cells (LAT). Once phosphorylated, LAT recruits the phospholipase Cγ (PLCγ) and SH2-containing adapters, such as the leukocyte protein of 76 kDa (SLP-76) and Grb2. PLCγ undergoes activating tyrosine phosphorylation and hydrolyzes the membrane phosphatidyl inositol 4,5-bisphosphate [PtdIns(4,5)P₂] to form the soluble inositol 1,4,5-trisphosphate (IP₃) and the membrane-bound diacylglycerol. These second messengers are responsible for intracellular calcium mobilization and protein kinase C activation, respectively [2]. The adapters SLP-76 and Grb2 recruit exchange factors, promoting the activation of the small GTPases Ras, Rac, and Rho. They regulate complex networks of signaling pathways, leading to the degranulation of preformed mediators and the de novo synthesis and release of eicosanoids, cytokines, and chemokines [33].

In RBL-2H3 cells, a complementary pathway is initiated by another PTK of the Src family, i.e. Fyn, which cooperates with the Lyn/Syk pathway in the propagation of FcεRI-mediated signals [34]. Upon receptor engagement, Fyn phosphorylates the molecular adapter Gab2, favoring membrane recruitment of the p85 regulatory subunit of phosphatidylinositol-3-OH kinase (PI3K). Once activated, PI3K catalyzes the formation of PtdIns(3,4,5)P₃ (PIP₃) which functions as the docking site for pleckstrin homology domain-containing proteins such as Bruton's tyrosine kinase and PLCγ.

It is unclear whether the Fyn pathway controls PI3K activation in human basophils [4]. Regardless, the PI3K

pathway plays an essential role in governing the production and release of different classes of mediators in basophils. Indeed, specific PI3K inhibitors affect degranulation as well as the generation of arachidonic acid metabolites and cytokine secretion [35, 36].

Negative Regulation of Fc ϵ RI-Mediated Signal Transduction

The degree of mast cell and basophil activation has to be precisely regulated to ensure a tightly constrained response.

Fc ϵ RI-mediated cell activation is counteracted by the action of inhibitory receptors constitutively or inducibly expressed on the surface of mast cells and basophils [5]. The extracellular domains of these receptors consist of either C-type lectin domains [e.g. mast cell-function-associated antigen (MAFA)] [37] or Ig-like domains [e.g. Fc γ RIIB, gp49B1, paired Ig-like receptor B (PIR-B), signal regulatory protein- α (SIRP α), and allergy inhibitor receptor 1 (Allergin1)] [38, 39].

The Ig-like inhibitory receptors are characterized by the presence of immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that, once phosphorylated, recruit and activate protein and lipid phosphatases [i.e. SH2 domain-containing protein tyrosine phosphatase (SHP) and SH2 domain-containing inositol-polyphosphate 5-phosphatase (SHIP)] [40]. For example, in human basophils, the coaggregation of Fc ϵ RI with the ITIM containing Fc γ RIIB induces the recruitment and activation of the tyrosine phosphatase SHP-1 to the phosphorylated ITIM with the consequent inhibition of Fc ϵ RI-mediated cell activation [41].

Thus, mechanistically, ITIM-bearing receptors suppress Fc ϵ RI-mediated signaling by promoting dephosphorylation events.

Fc ϵ RI engagement itself promotes dephosphorylation events that counteract PTK activation [5]. In RBL-2H3 cells, the phosphatases SHP-1, SHP-2, and SHIP-1 are tyrosine phosphorylated and activated upon their recruitment to the tyrosine-phosphorylated ITAM of the Fc ϵ RI β -chain [42, 43]. SHIP-1-deficient BMMCs exhibit enhanced degranulation and proinflammatory cytokine production compared to wild-type BMMCs [44].

Along the same line, a low expression of SHIP-1 was observed in 'hyper-releasable' basophils derived from highly allergic donors, and it was found to correlate with a higher sensitivity to stimulation [45]. On the other hand, SHIP-1 phosphorylation negatively correlates with

basophil releasability, suggesting a role for SHIP-1 as a negative regulator of IgE-mediated basophil functions [46].

In RBL-2H3 cells, SHIP-2, homolog of SHIP-1, negatively regulates degranulation and cytokine secretion, inhibiting Fyn-Gab2-dependent microtubule formation and the activation of the transcription factor GATA-1 [47].

Furthermore, in human mast cells, the absence of another lipid phosphatase, i.e. PTEN, leads to increased PIP₃ levels even in resting conditions. In PTEN-deficient cells, the enzymatic activity of c-Jun and p38 MAPKs is increased and is followed by a constitutive secretion of cytokines [48].

Interestingly, signaling molecules including PTKs and adapters also generate negative signals, contributing to the determination of the rate and the extent of functional mast cell responses [5]. Notably, Cbl proteins have emerged as a new family of negative regulators of Fc ϵ RI-mediated signals.

Cbl Family of Adapter Proteins

Structure and Function of Cbl Proteins

In mammals, 3 Cbl proteins encoded by separate genes have been identified: c-Cbl, Cbl-b, and Cbl-c (also known as Cbl-3) [6–8]. Among the 3 members of this family, c-Cbl and Cbl-b are expressed in hematopoietic cells, while Cbl-3 is expressed only in epithelial tissues.

All of these members are structurally characterized by the presence of highly conserved protein-protein interaction domains (fig. 1). The N-terminal tyrosine kinase-binding (TKB) domain consists of a 4-helix bundle, a calcium-binding EF hand, and a modified SH2 domain so named for its ability to bind to phosphotyrosine residues of receptor PTKs, including growth factor receptors such as EGFR and PDGFR as well as cytoplasmic tyrosine kinases such as the Syk/ZAP-70 family [49].

All Cbl proteins contain 2 additional protein-interacting domains in their N-terminal: a RING finger domain that catalyzes protein ubiquitination, interacting with Ub-conjugating enzymes [50, 51], and a proline-rich domain.

Moreover, c-Cbl and Cbl-b share additional regions in their C-terminal: an extensive proline-rich domain able to interact with Src family kinases and several adapter proteins, including CIN85/CMS; a region containing tyrosine residues that, once phosphorylated, promotes the interaction with SH2-domain containing proteins, and a

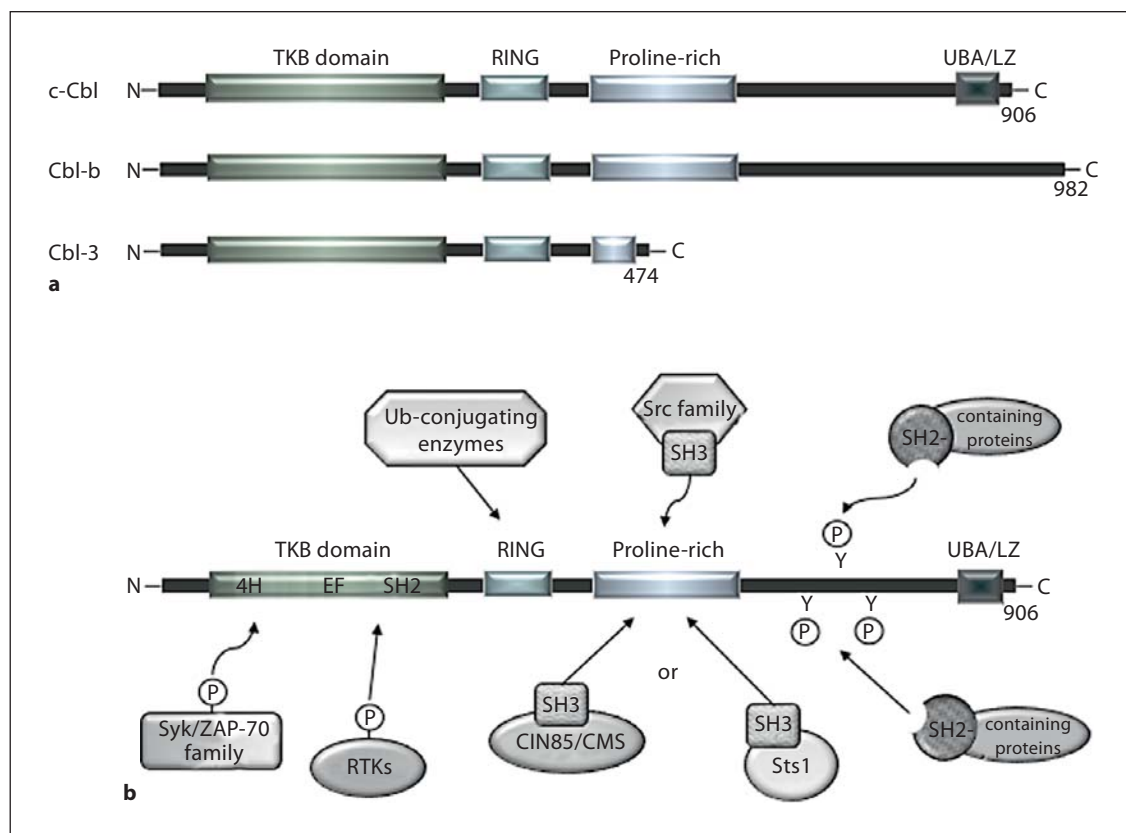


Fig. 1. Schematic representation of Cbl protein structure. **a** Protein domains of the 3 mammalian Cbl (Casitas B-lineage lymphoma) isoforms c-Cbl, Cbl-b, and Cbl-3. **b** The domains of c-Cbl and Cbl-b and their interactions with signaling proteins are outlined. The TKB domain mediates binding to phosphorylated tyrosines on receptor and cytoplasmic kinases. The RING finger domain recruits Ub-conjugating enzymes. The C-terminal proline-rich region is involved in the adapter functions of Cbl pro-

teins, serving as a docking site for several SH3-containing proteins. The C-terminal UBA-LZ domain plays a role in Cbl oligomerization and Ub binding. The region between the proline-rich and UBA-LZ domains contains several tyrosine residues that, once phosphorylated, promote the interaction with SH2-domain-containing proteins. CMS = Cas ligand with multiple SH3 domains; RTK = receptor tyrosine kinase.

region homologous to Ub-associated (UBA) and leucine zipper (LZ) domains that mediate Ub binding and intermolecular oligomerization, respectively.

The negative regulation of signal transduction by Cbl proteins depends on their ability to activate the Ub pathway [49].

Ubiquitination is a posttranslational reversible modification whereby Ub, a 76-amino acid globular peptide (8 kDa), is covalently attached to the lysine residue of acceptor proteins that are then targeted for degradation [52–54]. The reaction is catalyzed by a hierarchical set of 3 different enzymes, i.e. E1, E2, and E3 (fig. 2a). In an ATP-dependent reaction, Ub is bound to the Ub-activating enzyme (E1) through a thiol-ester bond. The activated Ub is successively accepted by the Ub-conjugating en-

zyme (E2) by transthiolation and is finally transferred to the substrate through the action of the Ub protein ligase (E3). This latter class of enzymes provides specificity to the Ub system being responsible for substrate recognition and Ub ligation to the target protein.

While a single gene encoding the E1 enzyme has been identified, several E2 and E3 enzymes exist in mammals and each is characterized by different domains deputed to Ub conjugation.

The Cbl family belongs to the E3 Ub ligases characterized by the presence of a RING finger domain that mediates the transfer of Ub to Cbl-interacting proteins [51] (fig. 2b).

c-Cbl ligase activity is regulated by the tyrosine phosphorylation of its linker domain located between the TKB

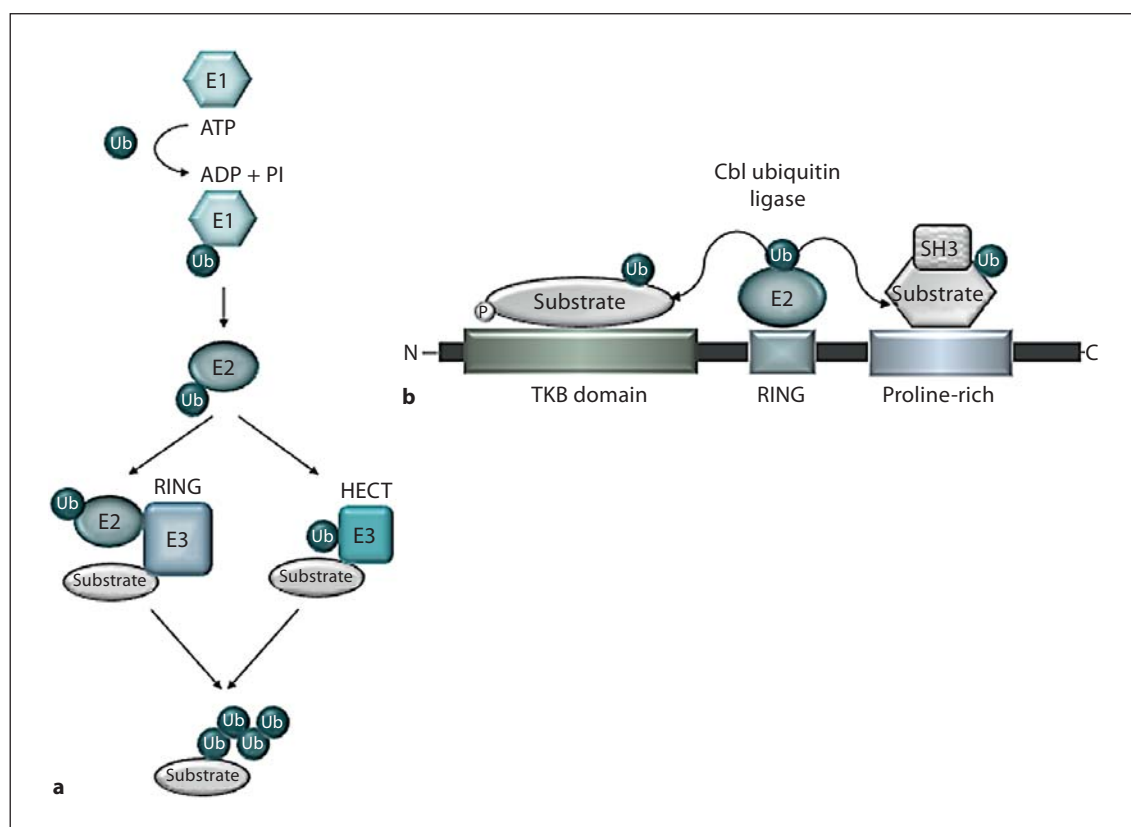


Fig. 2. The Ub pathway. **a** Schematic representation of the ubiquitination process. A hierarchical set of 3 types of enzymes is required for substrate ubiquitination: Ub-activating (E1), Ub-conjugating (E2), and Ub-protein ligase (E3) enzymes. E3 ligases of the RING finger and HECT domain classes are depicted. HECT = Homologous to the E6-AP carboxyl terminus. **b** Model

of Cbl ligase activity. Multiple motifs in Cbl proteins, such as the TKB and proline-rich domains, serve to recruit the substrates for ubiquitination. The Ub-conjugating enzyme (E2), upon interaction with the RING domain of the Ub ligase Cbl, transfers Ub to the target proteins.

and the RING finger. These phosphorylation events cause conformational changes in c-Cbl that are required for the release of the RING finger-associated E2, an essential step in Cbl-driven ubiquitination [55].

Ubiquitinated substrates are therefore targeted to different degradation pathways depending on the kind of ubiquitination itself. Polyubiquitin chains whereby Ub acts as a substrate for the attachment of further Ub molecules are commonly recognized as signals that target substrates to degradation by the 26S proteasome [52, 53, 56]. The attachment of a single Ub to 1 or more lysines of target proteins, i.e. mono- and multiubiquitination, respectively, can act as an internalization signal driving the transport of membrane receptors along the endocytic pathway towards a lysosomal compartment for degradation [57, 58].

In vitro, c-Cbl and Cbl-b show a similar ability to act as E3 Ub ligases toward a similar range of substrates.

However, unlike c-Cbl, ubiquitination by Cbl-b does not often result in substrate degradation but rather appears to affect protein localization [49].

c-Cbl and Cbl-b are both expressed on rodent and mouse mast cells and, upon FcεRI engagement, become tyrosine phosphorylated [59, 60] and translocate into lipid rafts [61, 62].

c-Cbl is also expressed on cultured human mast cells [63] and basophils [64] where it undergoes antigen-dependent phosphorylation.

Notably, the incubation for several days of peripheral blood basophils with a high concentration of IL-3 strongly upregulates Cbl levels [46].

Cbl-Mediated Regulation of FcεRI Endocytosis

In the past years, evidence has emerged that strongly supports the crucial role of Cbl family proteins in the

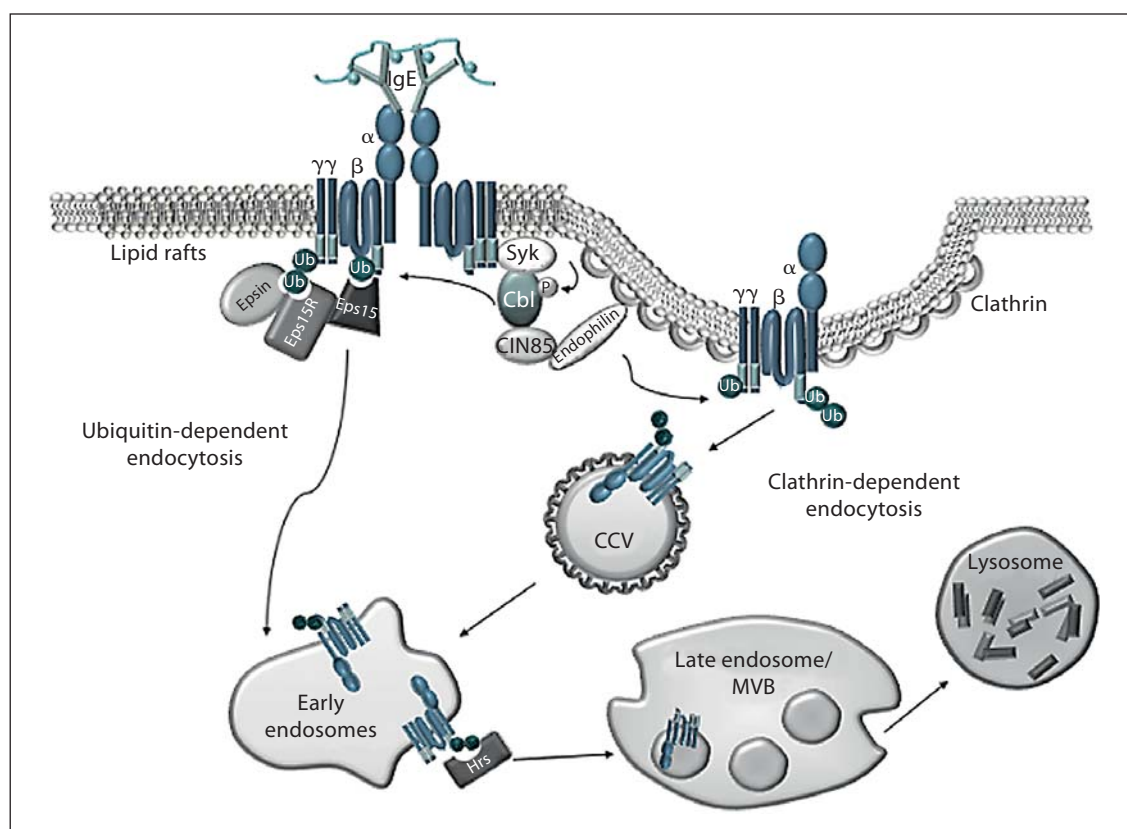


Fig. 3. Schematic model of ligand-induced FcεRI endocytosis. Engaged FcεRI complexes relocate into lipid rafts and undergo ubiquitination by the combined action of Syk and Cbl. Receptor multiubiquitination may act as an independent signal for receptor endocytosis allowing receptor interaction with Ub-binding adapters, such as Epsin, Eps15, and Eps15R, that promote receptor entry from the plasma membrane. Cbl also mediates the recruit-

ment of the CIN85/endophilin complex in the proximity of engaged receptors, whereby endophilin drives FcεRI clathrin-mediated endocytosis. Internalized receptor complexes are then sorted along the endocytic pathway and, through the action of Hrs, are targeted to the multivesicular bodies (MVBs) and to the lysosomes for degradation.

downregulation of immunoreceptors including FcεRI [9, 65, 66].

Our group firstly demonstrated that c-Cbl is the main E3 ligase responsible for FcεRI ubiquitination. After having established that FcεRI β and γ subunits undergo ubiquitination upon receptor aggregation in RBL-2H3 cells [67], we showed that the overexpression of wild-type c-Cbl, but not of a mutant form deleted in the RING finger domain, strongly increases antigen-induced FcεRI β and γ ubiquitination [9].

The concomitant evidence that both FcεRI and c-Cbl translocate into lipid rafts upon Ag stimulation [24, 61] prompted us to investigate the implication of a lipid raft environment in regulating Cbl-mediated FcεRI ubiquitination. We demonstrated that the recruitment of engaged FcεRI subunits into lipid rafts precedes their ubiqu-

itination and that the integrity of these membrane microdomains is required for receptor ubiquitination [68]. We further showed that a strong interdependence exists between lipid rafts and receptor endocytosis, which is in line with the findings of Fattakhova et al. [69], who demonstrated that aggregated FcεRI complexes remain associated with lipid rafts upon Ag-induced internalization.

All together, these data demonstrate that lipid raft-dependent ubiquitination acts as a signal for receptor entry from the plasma membrane.

In an effort to better clarify the function of c-Cbl-dependent FcεRI ubiquitination, we have also demonstrated that FcεRI β and γ subunits are mainly mono-ubiquitinated by c-Cbl at multiple sites, and we have provided evidence that this modification controls receptor internalization and sorting along the endocytic com-

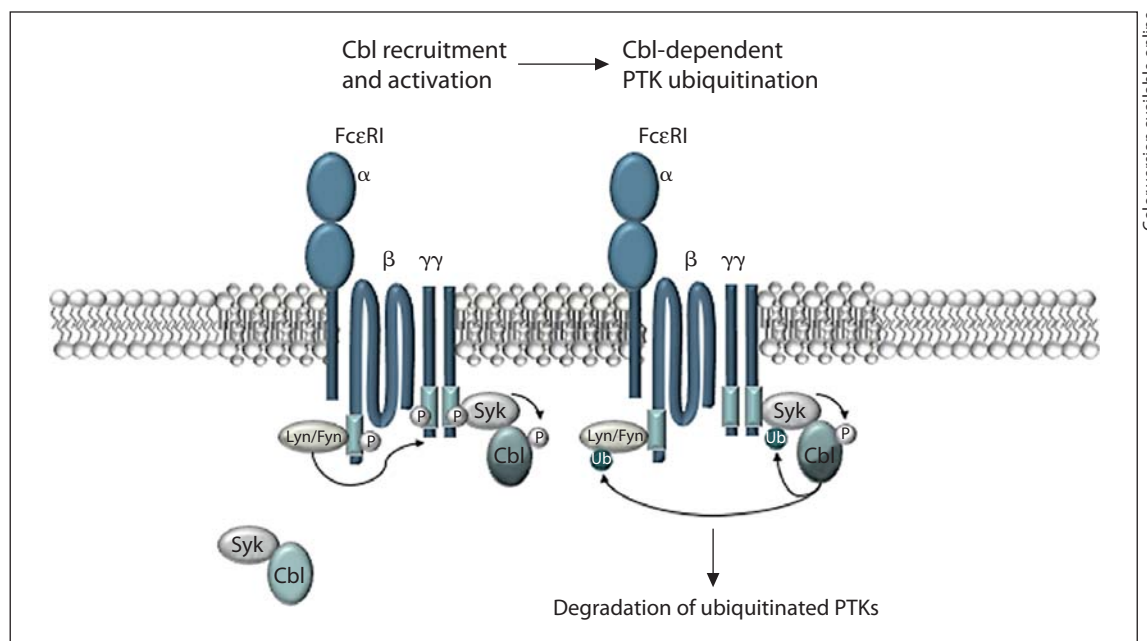


Fig. 4. Schematic model of PTK ubiquitination by Cbl. Upon Lyn-mediated receptor phosphorylation, a pre-formed Syk/Cbl complex binds to phosphorylated FcεRI subunits. Syk phosphorylates and activates Cbl which, in turn, ubiquitinates Syk and Src family PTKs.

partments through the interaction with Ub-binding adapters [68]. In particular, by means of siRNA, we have demonstrated the redundant role of the 3 Ub-binding adapters, i.e. Eps15, Eps15R, and Epsin, in receptor internalization as well as the critical role of Hrs in controlling the fate of internalized receptor complexes. Indeed, Hrs depletion retains ubiquitinated receptors in early endosomes and partially prevents their sorting into lysosomes [68].

All together, our data indicate that Cbl-dependent FcεRI ubiquitination regulates FcεRI internalization through the action of Ub-binding adapters and ensures the proper endocytic trafficking of an immune receptor to the lysosomal compartment where degradation of the complexes can take place (fig. 3).

Besides its role in driving receptor ubiquitination, c-Cbl can also control FcεRI downmodulation independently by its ligase activity through interaction with the multidomain protein CIN85 [11].

CIN85 is a broadly expressed adapter protein characterized by the presence of 3 SH3 domains and a proline-rich region, both involved in protein-protein interactions [70]. CIN85 binding to Cbl is mediated by its SH3 domains and is enhanced by ligand-induced tyrosine phosphorylation of Cbl, while the proline-rich region of CIN85

constitutively interacts with endophilin, a component of clathrin-coated pits [70].

In RBL-2H3 cells, we showed the presence of a Cbl/CIN85 interaction that increases upon antigen stimulation and demonstrated the role of this complex in driving FcεRI endocytosis [11]. Indeed, CIN85 overexpression results in a more rapid receptor internalization, entry into early endosomes, and delivery to a lysosomal compartment for degradation. In the same study, we also demonstrated an impairment of FcεRI-mediated degranulation in CIN85 transfectants, suggesting that the accelerated FcεRI internalization may contribute to dampening cell functional responses.

Based on our result, we propose a model in which Cbl, recruited to the membrane and activated upon FcεRI stimulation, regulates endocytosis of the engaged receptor complexes (fig. 3).

Cbl-mediated receptor ubiquitination provides a signal for FcεRI entry and sorting into endocytic compartments where the interaction with Hrs promotes its transfer into lysosomes for degradation. On the other hand, Cbl works as an adapter inducing the recruitment of the CIN85/endophilin complex to the plasma membrane where it drives the clathrin-dependent endocytosis of engaged FcεRI complexes.

Although not as extensively studied as in RBL-2H3 cells, evidence indicates that a role for Cbl proteins in FcεRI downregulation can also be envisaged in mouse mast cells.

Loss of Cbl-b, but not of c-Cbl, retards FcεRI internalization in BMMCs [71], but Cbl-b ligase activity is dispensable for receptor downregulation [72].

In conclusion, while in RBL-2H3 cells c-Cbl plays a major role in receptor downmodulation, both through its ligase activity and through its adapter function, in mouse mast cells the main function of Cbl-b is to work as an adapter protein.

The role of Cbl proteins in regulating FcεRI surface expression in human mast cells and basophils has not yet been investigated.

Cbl-Dependent Regulation of PTK Expression and Function

Cbl proteins play an additional negative role in controlling intracellular signaling by promoting the ubiquitination and degradation of Src and Syk family PTKs [9, 10, 64, 66, 73–75].

Syk and c-Cbl are constitutively associated in RBL-2H3 cells [59]. Notably, c-Cbl overexpression inhibits Syk activity and FcεRI-mediated serotonin release [76].

We have contributed to the elucidation of the molecular mechanism underlying the negative role of Cbl; we showed that c-Cbl is the E3 ligase responsible for Syk ubiquitination that preferentially affects phosphorylated and activated forms of the kinase, promoting their degradation and limiting signal propagation [9]. Our results are in line with the findings of Sada et al. [77] showing that a mutant of Syk unable to bind to Cbl enhances mast cell degranulation.

Thus, upon receptor ligation, the Syk/Cbl complex is recruited to the phosphorylated FcεRI subunits where Syk phosphorylates and activates c-Cbl which, in turn, ubiquitinates activated Syk (fig. 4).

Also in human basophils, antigen or anti-IgE-induced FcεRI stimulation promotes Syk ubiquitination together with a progressive proteasome-dependent loss of Syk protein [66]. An antigen-dependent interaction between Syk and tyrosine phosphorylated Cbl was also observed, suggesting the role of Cbl in targeting Syk ubiquitination and degradation. Moreover, IgE-independent stimuli are able to induce c-Cbl phosphorylation and Syk degradation, resulting in an impaired functional response [78]. However, additional research is required in order to demonstrate a formal role of Cbl proteins in promoting Syk ubiquitination and degradation in human basophils and mast cells.

In RBL-2H3 cells, Cbl proteins can also ubiquitinate Lyn and Fyn following antigen stimulation, further contributing to limiting FcεRI-mediated signal transduction and mast cell functions [10, 62]. Notably, Cbl proteins preferentially ubiquitinated the active forms of Lyn [10], supporting our previous finding that ubiquitination limits signal propagation targeting activated PTKs for degradation. Moreover, overexpression of the lipid raft-anchored forms of c-Cbl or Cbl-b proteins reduced FcεRI-mediated cell activation through the enhanced ubiquitination and degradation of Lyn or Gab2, respectively [10, 62].

Thus, overexpression studies in RBL-2H3 cells have shown that both c-Cbl and Cbl-b play negative regulatory roles in FcεRI-mediated signaling.

c-Cbl and Cbl-b knockout (KO) mice were useful in the highlighting of redundant or distinct roles of the 2 adapter proteins. In Cbl-b-deficient BMMCs FcεRI-mediated phosphorylation, Ca²⁺ mobilization, histamine release, and the induction of proinflammatory cytokines were increased compared to wild-type and c-Cbl KO mice [60, 71]. These results are in line with recent findings on BMMCs derived from knock-in mice overexpressing a mutant form of Cbl-b with a loss-of-function mutation in the RING finger domain [72]. Compared to wild-type mice, mast cells derived from Cbl-b RING finger mutant mice showed higher levels of total protein phosphorylation and cytokine production. However, upon antigen stimulation, the RING finger mutant mast cells do not produce the very high levels of TNF-α, IL-6, and MCP-1 evident in Cbl-b KO cultures. Thus, the ability of Cbl-b to function as a negative regulator of the FcεRI signaling responsible for inflammatory cytokine production is largely independent of the RING finger domain [72].

Regarding the molecular basis of the different biological effects exerted *in vivo* by c-Cbl and Cbl-b, additional experiments are required. In fact, since *in vitro* experiments have argued that c-Cbl and Cbl-b share the same regulatory role in FcεRI-mediated signals, the differences observed *in vivo* are probably due to higher expression levels of Cbl-b over c-Cbl in BMMCs, as reported by Gustin et al. [71].

Whether c-Cbl and Cbl-b proteins differentially regulate the secretion of preformed and *de novo* synthesized mediators, including eicosanoids and cytokines, also requires further investigation.

All together, the data obtained in RBL-2H3 cells and in BMMCs support the requirement of Cbl protein ligase activity for the negative regulation of FcεRI-induced signal propagation.

Cbl-Mediated Regulation of PTKs in Unstimulated Basophils and Mast Cells

The evidence collected over the past years has demonstrated that human basophils show a wide variability in the rate of FcεRI-mediated degranulation and that, among them, a small percentage of basophils fail to release histamine after FcεRI cross-linking. This unresponsiveness has been linked to lower levels of Lyn and to the absence of Syk, despite a normal level of their mRNA, suggesting the action of a posttranslational mechanism responsible for PTK degradation [31, 32]. This condition is reversible since the donors can fluctuate between the nonreleaser and releaser phenotypes with a concomitant change of Syk expression [31], and unresponsiveness can be overcome by IL-3 treatment [79].

Although differences in Cbl protein levels between releaser and nonreleaser basophils have not been observed, it is likely that alterations in Cbl ligase activity contribute to Syk instability in the nonreleaser phenotype. In this regard, an involvement of the Ub-proteasome pathway has been reported [80] and may contribute to the regulation of Syk levels in unstimulated basophils isolated from nonreleaser donors. Moreover, we have recently demonstrated that Cbl ligase activity is influenced by the relative protein abundance of specific positive and negative regulators also in unstimulated RBL-2H3 cells [81].

c-Cbl ligase activity is negatively regulated by interaction with different families of scaffold proteins [82], including the suppressor of T-cell receptor signaling 1 (Sts1) which, through its SH3 domain, binds the Cbl proline-rich domain [83]. We have demonstrated that CIN85 competes with Sts1 for Cbl binding and that CIN85 overexpression results in a positive regulation of Cbl ligase activity, promoting antigen-independent Syk ubiquitination and degradation with consequent functional defects [81].

Human basophils also express CIN85 [84]; thus, it will be important to address in the future whether any alteration of this molecular machinery may give rise to the nonreleaser phenotype.

Concluding Remarks and Perspectives

Mast cell and basophil activation through the high-affinity receptor for IgE is regulated by a balance of positive and negative signals.

Studies carried out with rat and murine cell lines have clearly demonstrated that both c-Cbl and Cbl-b negatively regulate FcεRI-mediated activation.

We have provided an overview of the molecular mechanisms through which Cbl proteins control the intensity and duration of FcεRI-mediated signals.

Cbl-dependent FcεRI downmodulation occurs mainly by means of Cbl Ub ligase activity that promotes receptor β and γ multiubiquitination, providing signals for receptor internalization and sorting into lysosomal compartments for degradation. On the other hand, Cbl proteins allow the downmodulation of engaged FcεRI through a pathway that is functionally separable from E3 ligase activity, but it depends on the Cbl interaction with adapters involved in clathrin-mediated endocytosis. Remarkably, Cbl proteins also negatively regulate the expression level of PTKs, including Lyn and Syk, by the selective ubiquitination and degradation of the activated kinase form.

Given the role of Cbl proteins as negative regulators of mast cell and basophil functions, they are candidate molecules for future application-oriented research aimed at developing gene therapy methods to treat allergic diseases.

Along these lines, our challenge is to formally demonstrate that c-Cbl and/or Cbl-b negatively regulates FcεRI surface expression and Syk protein levels also in human mast cells and basophils in order to use this knowledge to selectively overexpress Cbl proteins or increase their catalytic activity in the basophils and mast cells of allergic patients. This strategy has the potential to complement other therapies based on the neutralization of IgE or the pharmacological inhibition of Syk.

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