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Cbl exposes its RING finger

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Abstract

The Cbl family of RING finger ubiquitin ligases regulates signaling in many systems. Two new studies provide a structural basis for how phosphorylation of a specific tyrosine in the Cbl proteins enhances their ubiquitin ligase activity, giving insight into how ubiquitination by Cbl proteins is restricted to specific substrates.

Covalent attachment of ubiquitin to a substrate proceeds by means of a three-enzyme cascade initiated by the ATP-dependent attachment of the ubiquitin molecule to the ubiquitin-activating enzyme (E1). This is followed by the transfer of the ubiquitin molecule, through a transesterification reaction, to a ubiquitin-conjugating enzyme (E2) and, subsequently, by the interaction of the E2 with a ubiquitin ligase (E3), which facilitates the transfer of the ubiquitin molecule to the substrate¹. RING finger proteins constitute the majority of E3s, and accordingly, they are fundamental regulators of many crucial cellular processes^{2,3}. They show remarkable specificity for substrates *in vivo*, but when bacterial recombinant E3 proteins are studied *in vitro*, most have measureable constitutive E3 activity^{1–4}. The structural mechanisms that regulate the E3 activity of the RING finger proteins to allow the temporal and spatial restriction of ubiquitination to specific substrates *in vivo* is a fundamental question that is addressed by two recent studies of the Cbl RING finger E3s^{5,6}.

Cbl was first identified as the cellular homolog of the transforming gene of the Casitas B lymphoma murine retrovirus⁷. Cbl proteins have been found throughout the meta-zoans, with putative *Cbl* genes identified in sea urchin and *Dictyostelium discoideum* (Fig. 1). They all contain an N-terminal tyrosine kinase binding domain (TKB), a RING finger domain and a short conserved α -helical region, known as the linker region, between the TKB and the RING finger domains (Fig. 1). The TKB is a phosphotyrosine binding domain found only in Cbl proteins, composed of a four-helix bundle, a calcium-binding EF hand and a variant SH2 domain^{8,9}. Multiple binding motifs that interact through distinct mechanisms allow Cbl proteins to bind to many different proteins and thereby function as negative regulators of signaling by receptor tyrosine kinases (RTKs), non-receptor tyrosine kinases and kinase-associated receptors (for example, T-cell and B-cell receptors)⁷. Mutations leading to the loss of function in Cbl have been found in approximately 5% of myeloid neoplasms¹⁰, and

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loss-of-function mutations of *CBLB* have been implicated in autoimmune diseases such as type 1 diabetes and multiple sclerosis^{11,12}.

Crucial to understanding the role of Cbl proteins as negative regulators of signal transduction was the demonstration that the Cbl proteins are RING finger E3s that ubiquitinate activated RTKs and target them for degradation^{13–15}. Upon activation and autophosphorylation of the kinases, the Cbl proteins bind directly (through their TKB)^{8,14,16} or indirectly (through adaptor-dependent mechanisms)^{17,18} to phosphotyrosines on the substrate RTK. Phosphorylation of a conserved tyrosine in the linker preceding the RING finger has been shown to be essential for activation of the E3 activity of all Cbl proteins both *in vivo* and *in vitro*, potentially through a conformational change^{14,19,20}. Thus, activation of the RTK serves to create a phosphotyrosine-based docking site on the RTK for the Cbl proteins and to phosphorylate and stimulate the E3 activity of the Cbl proteins.

Two studies have now shed light on the mechanism for phosphorylation-induced activation of CBL⁵ and CBLB⁶. In this issue, Dou *et al.*⁵ present the crystal structures of the CBL N terminus (including the TKB domain, linker and RING finger domain), the N terminus bound to a substrate phosphopeptide through the TKB, and the N terminus phosphorylated on the linker tyrosine (pTyr371) bound to a substrate phosphopeptide and an E2. In the absence of substrate peptide binding or phosphorylation of the linker tyrosine, the TKB domain forms a compact structure with the RING finger that masks the E2 binding sites. Binding of a phosphopeptide to the TKB domain induces a rotation, partially exposing the E2 binding domain. Upon phosphorylation of the linker tyrosine, there is a marked rotation of the linker domain, which fully exposes the E2 binding surface of the RING finger and simultaneously brings the E2 closer to the substrate bound to the TKB domain. In studies of CBLB using NMR and small-angle X-ray scattering measurements, Kobashigawa *et al.*⁶ similarly found that the TKB domain of CBLB packs tightly against the RING finger when the linker tyrosine is unphosphorylated, and that phosphorylation of the linker tyrosine results in unfolding of the Cbl protein to unmask the RING finger E2 binding surface.

Interestingly, these solution studies revealed an equilibrium between the tightly folded unphosphorylated protein and a partially unfolded structure, even in the absence of substrate binding, although the equilibrium favored the closed state⁶. In the previously reported crystal structure of unphosphorylated CBL in complex with the E2 UbcH7, the linker tyrosine was bound to the TKB and was thus not readily available for phosphorylation²¹. Importantly, both of the new studies reveal that the unphosphorylated protein exists in both a closed-structure form and a partially unfolded state, which would make the tyrosine accessible for phosphorylation. Whether the equilibrium seen by Kobashigawa *et al.* between the closed and partially unfolded form is perturbed by the binding of the substrate to the TKB remains to be determined. Phosphorylation of the linker tyrosine residue markedly increased the E2 binding affinity to the RING finger of either CBL⁵ or CBLB⁶. Concomitant with this increased affinity, there was increased E3 activity by the phosphorylated proteins in both studies^{5,6}.

Together, these studies suggest an intriguing model for Cbl protein regulation (Fig. 2). The Cbl proteins are kept in an inactive state by masking of the E2 binding site by the TKB–

RING finger interaction until binding of a phosphorylated substrate through the TKB and phosphorylation of the linker tyrosine promote full activation of the Cbl E3 function. Intrinsic to this model is an explanation of the specificity of the Cbl proteins for activated kinase substrates, with which they interact through the TKB domain. A recent study suggested that the phosphorylation of the linker tyrosine of the third mammalian Cbl protein, Cbl-c, enhanced E3 activity by allowing for more rapid turnover of the E2 (and thus for greater processivity of the ubiquitin reaction)²⁰. How this observation fits with the data from the current studies requires further investigation. In addition, Cbl proteins can interact with and ubiquitinate proteins in a TKB-independent manner. For example, Cbl-b binds, through C-terminal proline-rich regions, to an SH3 domain of the p85 subunit of phosphatidylinositol-3-kinase and ubiquitinates the p85 subunit, thereby preventing the activation of the co-stimulatory pathway in T cells^{22,23}. Whether the E3 activity in such cases depends on phosphorylation of the linker tyrosine and—if so—how it is mediated remain to be determined.

Interestingly, the HECT E3s ITCH and SMURF2 adopt an autoinhibited structure that is relieved either by serine and threonine phosphorylation or by binding of an activating protein, respectively^{24,25}. Thus, the relief of E3 autoinhibition by either covalent modification or protein binding as a means to temporally regulate E3 activity may be a general mechanism whose scope is just now becoming fully appreciated.

The detailed understanding of the structural changes that occur in Cbl proteins upon phosphorylation of the linker tyrosine could allow for the structure-based development of inhibitors of Cbl E3 activity. In animal studies, the loss of Cbl-b results in enhanced anti-tumor immune responses^{26,27}, so inhibitors of Cbl proteins may stimulate the host immune response to tumors or enhance other immunity-based therapies. Similarly, it is conceivable that inhibition of Cbl protein function might be beneficial in other immune-deficient states. A number of intracellular bacteria enter cells by endocytic mechanisms that are dependent on Cbl proteins, and here again, inhibition of Cbl function might be beneficial to the control of such infections^{28,29}. Intriguingly, it is also conceivable that activators of the Cbl proteins could be designed to restore function where it has been lost. For example, in myeloid neoplasms, the linker tyrosine of Cbl is frequently mutated, resulting in the loss of E3 activity¹⁰. A small molecule that could bind to the linker and mimic the phosphorylated linker tyrosine could potentially restore activity of the Cbl protein and inhibit tumor growth. Considering the conserved nature of Cbl proteins throughout the metazoans, the growing list of interacting partners and the association of Cbl proteins with cancers, understanding the regulation of these proteins will no doubt have a substantial impact on the design of therapies targeted not only to Cbl proteins, but perhaps also to other RING finger E3s.

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Figure 1.

The Cbl proteins. There are three mammalian Cbl proteins⁷, encoded by separate genes: Cbl (also known as c-Cbl, CBL2 or RNF55), Cbl-b (also known as RNF56) and Cbl-c (also known as Cbl-3, Cbl-SL or RNF57). D-Cbl_L and D-Cbl_S are the long and short spliced isoforms, respectively, of the *Drosophila melanogaster* Cbl protein. Sli-1 is the *Caenorhabditis elegans* Cbl protein. All Cbl proteins contain a highly conserved N-terminal TKB domain, a linker region (L) and a C3HC4 RING finger (RF)⁷. The C termini of the Cbl proteins contain tyrosines (Y) that can become phosphorylated and mediate interactions with SH2-containing proteins. The C termini also contain proline-rich regions (P) that mediate interactions with SH3-containing proteins⁷. The longer forms of Cbl proteins have a ubiquitin-associated (UBA) domain at the C terminus⁷. 4H, four-helix bundle.

Figure 2.

Model of Cbl function. (1) Prior to interaction with a substrate, Cbl proteins exist in an inactive closed conformation in which the TKB domain interacts with the RING finger in a tightly packed structure that blocks E2 binding. This form is in equilibrium with a partially unfolded structure. The ubiquitin-charged E2 can bind to the partially unfolded Cbl RING finger with low affinity, but the Cbl protein is inactive. (2) Upon activation and autophosphorylation of the RTK, the Cbl protein interacts with a phosphorylated tyrosine on the RTK through the TKB. This interaction may either stabilize or select for the partially relaxed structure. (3) The Cbl protein is phosphorylated on the linker tyrosine, causing a rotation of the linker region and thus exposing the RING finger for higher-affinity binding of the ubiquitin-charged E2 and bringing the E2 into close proximity to the substrate. (4) The Cbl protein is now active and enhances transfer of ubiquitin from the bound E2 to the substrate. (5) The ubiquitination of the RTK leads to trafficking of the RTK through the endocytic system to the lysosome for degradation. RF, RING finger; P, proline-rich regions; UBA, ubiquitin-associated domain; Y, linker tyrosine residue; Ub, ubiquitin; PO₄, phosphate groups. The linker is depicted as a helix, and the linker tyrosine is indicated.