Substrate Binding, not Phosphorylation, Activates CBL Poly-ubiquitination Activity

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ABSTRACT

Casitas B-lineage lymphoma (CBL) is a family of RING-type E3 ligases that target proteins for degradation via the proteasomal pathway. CBL-b specifically functions as a negative effector of T cell activation by down regulating the T cell receptor, whereas c-CBL interacts with various receptor tyrosine kinases involved in cell signaling and activation, targeting them for degradation. Therefore, the CBL family regulates the biology of immune cells and targeting CBL-b or c-CBL is a potential therapeutic strategy for the treatment of cancer, infection, or autoimmune diseases.

In this study, we used TR-FRET (Time-Resolved Fluorescence Resonance Energy Transfer) to uncover new mechanistic aspects of CBL activation. Our assays were designed and optimized using purified components (E1/E2/c-CBL or CBL-b E3 ligase and kinase substrate Tyro3 or Axl) to recreate the polyubiquitination cascade *in vitro*. The assays use a Europium cryptate-labeled Ubiquitin (donor) and a Cy5-labeled Ubiquitin (acceptor) to complete the TR-FRET pairing. The technique measures poly-ubiquitination, not mono-ubiquitination.

Validation of the assay indicated that the presence of a kinase substrate (Tyro3 or Axl) activates CBL-b and c-CBL, which are known to display a similar mechanism of activation. Importantly, our assay design allowed us to show that known CBL-b inhibitor CBL-B-IN-1 is not strictly selective toward CBL-b but it also affects c-CBL E3 ligase activity to similar extend since the IC_{50} of the compound for c-CBL and CBL-b differ by only 2-fold. Finally, it has been proposed that CBL-b phosphorylation at Y106, Y133, and Y363 by Tyro3 kinase is required for its activation. Indeed, in our assay, we observed that CBL-b mutation at Y363F decreases Tyro3 ubiquitination drastically. Surprisingly, phospho-mimetic mutation Y363E did not restore CBL-b-mediated ubiquitination of Tyro3, indicating that substrate binding close to Y363 is the predominant requirement for CBL activation. The weak E3 ligase activity of CBL-b Y363E mutant was insensitive to CBL-B-IN-1 treatment. Our data indicates that no significant difference in the parameters of substrate ubiquitination exists between CBL-b and c-CBL, and that ubiquitination of receptor tyrosine kinase Tyro3 by either c-CBL or CBL-b depends on the kinase binding to CBL but not on the phosphorylation of CBL.

BACKGROUND & ASSAY PRINCIPLE

Aberrant signaling mediated by mutated or overexpressed receptor tyrosine kinases (RTKs) is present in many human cancers. The E3 ligases c-CBL and CBL-b restrain RTK signaling through the ubiquitination and subsequent proteasomal or lysosomal degradation of RTKs, leading to signal termination. Therefore, engaging CBL function is an attractive therapeutic strategy in oncology.

The two CBL proteins contain a highly conserved N-terminal tyrosine kinase-binding domain (TKBD) followed by a linker helix region (LHR) and a RING domain. Their E3 ligase activity is positively regulated by phosphorylation of a conserved tyrosine (Tyr363 in CBL-b and Tyr371 in c-CBL) located within LHR. Under basal conditions, this tyrosine is anchored to the TKBD and constrains the RING domain in an inactive conformation. Upon growth factor stimulation, CBL is recruited to the activated RTK, which phosphorylates several CBL tyrosine residues including Tyr363/371, leading to conformational changes and E3 activation. Naturally occurring mutations within the LHR and RING domains, with Tyr363/371 as the major hotspot, have dominant-negative effects and are associated with cancer progression in the clinic.

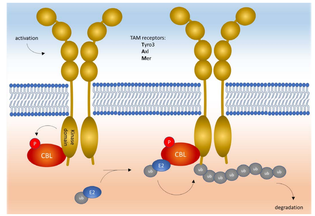


Figure 1. Illustration of CBL activation. CBL proteins are recruited to activated RTKs, and mediate RTK ubiquitination and subsequent downregulation.

This study reveals new mechanistic insights into the stimulation of c-CBL and CBL-b E3 ligase poly-ubiquitination activity using recombinant tyrosine kinase domains as substrate.

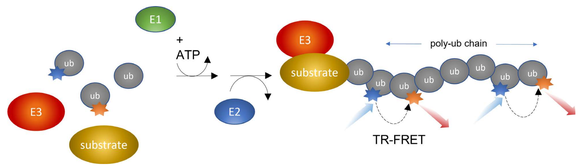


Figure 2. Illustration of the assay principle. The CBL-b and c-CBL-driven Intrachain TR-FRET Assay Kits were developed to measure CBL E3 ligase activity toward a kinase substrate in a homogeneous, 384 reaction format. A Europium-Ubiquitin Donor and a Cy5-Ubiquitin Acceptor form the TR-FRET pairing. Since both the fluorescence donor and acceptor are incorporated into poly-ubiquitin chains, this assay does not detect mono-ubiquitination. The FRET-based format requires no time-consuming washing steps, making it especially suitable for high-throughput applications as well as real-time analyses of poly-ubiquitination.

RESULTS

CBL-b-drives poly-ubiquitination of Tyro3

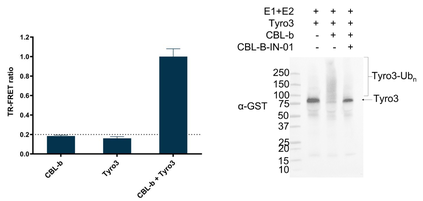


Figure 3. CBL-b-mediated poly-ubiquitination of Tyro3 was measured in the presence of 40 nM E1, 100 nM E2, 25 nM kinase, and 12.5 nM CBL-b for 30 minutes at room temperature. Left: Poly-ubiquitination of Tyro3 by CBL-b was monitored using the intrachain TR-FRET assay. Right: CBL-b-mediated poly-ubiquitination of Tyro3 was analyzed by Western blot.

CBL-b Y363 mutation reduces Tyro3 poly-ubiquitination

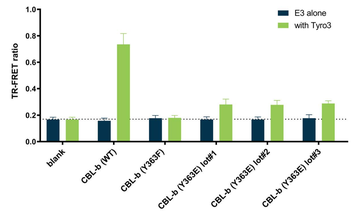
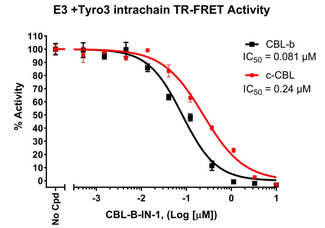
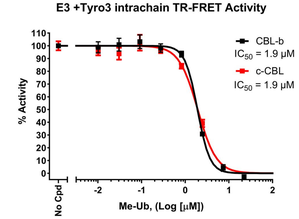


Figure 4. CBL-b-mediated poly-ubiquitination of Tyro3 was measured in the presence of 40 nM E1, 100 nM E2, 25 nM kinase, and 12.5 nM CBL-b wild-type or mutant for 30 minutes at room temperature. Substitution of Tyrosine 363 (Y363) by phenylalanine (F) or by phospho-mimetic glutamic acid (E) reduced substrate poly-ubiquitination.

E3 +Tyro3 intrachain TR-FRET Activity



E3 +Tyro3 intrachain TR-FRET Activity



Both Tyro3 and AXL are poly-ubiquitinated by CBL-b and c-CBL

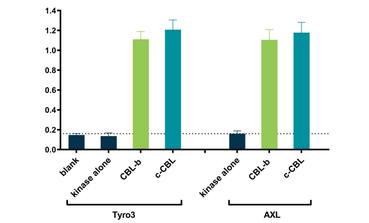


Figure 5. CBL-mediated poly-ubiquitination of Tyro3 and AXL was measured in the presence of 40 nM E1, 100 nM E2, 25 nM kinase, and 12.5 nM CBL.

CBL-b and c-CBL displayed similar reaction rates

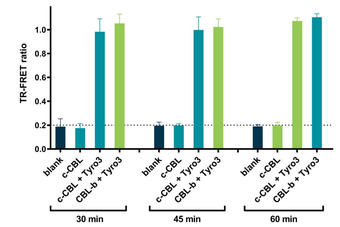
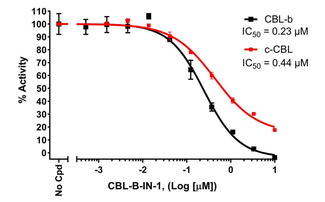


Figure 6. Real-time analysis of CBL-mediated poly-ubiquitination of Tyro3 was done in the presence of 40 nM E1, 100 nM E2 and 12.5 nM c-CBL or CBL-b. Data shown only for the indicated time points. Poly-ubiquitination of Tyro3 was maximal starting at 30 minutes.

E3 +AXL intrachain TR-FRET Activity



E3 +AXL intrachain TR-FRET Activity

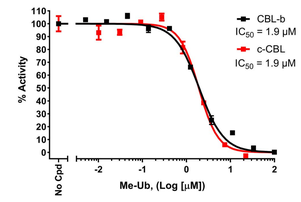


Figure 9. Inhibition of CBL-b and c-CBL-driven ubiquitination of Tyro3 and AXL by CBL-B-IN-1 and Methyl-Ubiquitin assessed using TR-FRET assays. CBL-b or c-CBL-dependent ubiquitination of Tyro3 or AXL was measured in the presence of increasing concentrations of CBL-B-IN-1 inhibitor (MedChem Express #HY-136339) and methylated Ubiquitin. Inhibitors IC_{50} was determined using CBL-b and c-CBL-driven kinase ubiquitination Intrachain TR-FRET Assay Kits (BPS Bioscience #78388;; #78821; #78408; #78823).

CBL-B-IN-01 inhibits CBL-b and c-CBL activity equally well

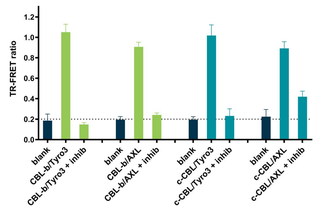


Figure 7. CBL-mediated poly-ubiquitination of Tyro3 or AXL was measured in the presence of 40 nM E1, 100 nM E2 and 12.5 nM c-CBL or CBL-b for 30 minutes at room temperature, with or without 1 µM CBL-B-IN-01.

Weak activity of CBL-b Y363E is insensitive to CBL-B-IN-01

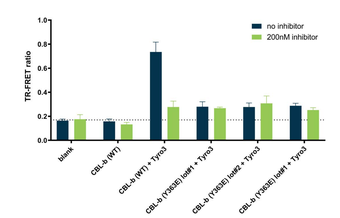


Figure 8. CBL-b-mediated poly-ubiquitination of Tyro3 was measured in the presence of 40 nM E1, 100 nM E2 and 12.5 nM CBL-b (E3) for 30 minutes at room temperature, with or without 200 nM CBL-B-IN-01.

CONCLUSIONS

- ◊ CBL-b and c-CBL display similar activation patterns for ubiquitination of protein tyrosine kinase (TK) substrates.
- ◊ Phosphorylation of Tyrosine 363 (Y363) in CBL-b (which corresponds to Y371 in c-CBL) is critical for TK binding and subsequent poly-ubiquitination of the kinase.
- ◊ CBL-b mutation Y363F drastically decreases Tyro3 ubiquitination.
- ◊ Phospho-mimetic mutation Y363E did not restore CBL-b-mediated ubiquitination of Tyro3, indicating that binding a kinase substrate close to Y363 is the predominant requirement for its polyubiquitination.
- ◊ Weak but noticeable poly-ubiquitination of Tyro3 was observed using CBL-b Y363E mutant, which was not inhibited by CBL-B-IN-1 treatment.
- ◊ CBL-B-IN-01 inhibits both CBL-b and c-CBL E3 ligase activity.

Homogeneous Intrachain TR-FRET Assays:

- ◊ Measure CBL-mediated polyubiquitination of receptor protein tyrosine kinases.
- ◊ Are suitable for High Throughput Drug Screening applications and quickly determine compound IC_{50}.
- ◊ Enable real-time kinetics analyses.