Molecular Pathways: Cbl Proteins in Tumorigenesis and Antitumor Immunity—Opportunities for Cancer Treatment

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Abstract

The Cbl proteins are a family of ubiquitin ligases (E3s) that regulate signaling through many tyrosine kinase–dependent pathways. A predominant function is to negatively regulate receptor tyrosine kinase (RTK) signaling by ubiquitination of active RTKs, targeting them for trafficking to the lysosome for degradation. Also, Cbl-mediated ubiquitination can regulate signaling protein function by altered cellular localization of proteins without degradation. In addition to their role as E3s, Cbl proteins play a positive role in signaling by acting as adaptor proteins that can recruit signaling molecules to the active RTKs. Cbl-b, a second family member, negatively regulates the costimulatory pathway of CD8 T cells and also negatively regulates natural killer cell function. The different functions of Cbl proteins and their roles both in the development of cancer and the regulation of immune responses provide multiple therapeutic opportunities. Mutations in Cbl that inactivate the negative E3 function while maintaining the positive adaptor function have been described in approximately 5% of myeloid neoplasms. An improved understanding of how the signaling pathways [e.g., Fms-like tyrosine kinase 3 (Flt3), PI3K, and signal transducer and activator of transcription (Stat)] are dysregulated by these mutations in Cbl has helped to identify potential targets for therapy of myeloid neoplasms. Conversely, the loss of Cbl-b leads to increased adaptive and innate antitumor immunity, suggesting that inhibiting Cbl-b may be a means to increase antitumor immunity across a wide variety of tumors. Thus, targeting the pathways regulated by Cbl proteins may provide attractive opportunities for treating cancer.

Background

Cbl proteins are a highly conserved family of ubiquitin ligases (E3s) primarily found in metazoans that negatively regulate signal transduction through many tyrosine kinases–dependent pathways (comprehensively reviewed in ref. 1). Mutations in Cbl proteins contribute to the pathogenesis of cancer by dysregulating receptor tyrosine kinase (RTK) signaling pathways. Further, Cbl-b, the second mammalian Cbl protein, negatively regulates T-cell and natural killer (NK)-cell antitumor function. Together, the data emerging about how Cbl proteins contribute to the pathogenesis of cancer and how they regulate antitumor immunity may provide a number of attractive approaches to cancer treatment.

The Cbl proteins as regulators of signaling

First identified as the cellular homologues of the v-Cbl–transforming gene of the Cas NS-1 murine retrovirus, Cbl proteins have been found throughout metazoans (2). There are three mammalian Cbl proteins: Cbl (a.k.a., c-Cbl; CBL2; RNF55), Cbl-b (a.k.a., RNF57), and Cbl-c (a.k.a., Cbl-3, Cbl-SL, RNF57; ref. 2). Cbl proteins are characterized by a highly conserved N-terminal tyrosine kinase binding (TKB) domain and a C3HC4 RING finger (RF) that is the catalytic domain for the E3 activity (2). These two domains are separated by a highly conserved alpha-helical linker region that is critical to the regulation of Cbl E3 function. The Cbl proteins vary more in the C-terminus which contains motifs (e.g., proline-rich domains, tyrosines which become phosphorylated, and an ubiquitin-associated domain) which mediate a broad array of protein interactions with signaling molecules (3). The Cbl proteins are tyrosine phosphorylated upon activation of a variety of growth factor receptors, and they associate with many proteins containing SH2 and SH3 domains (reviewed in refs. 4–6). These diverse interactions modulate signaling both negatively and positively through many pathways (1, 4–6).

The E3 activity of Cbl proteins is critical to the negative regulation of signaling by activated RTKs (Fig. 1A). The covalent modification of proteins by ubiquitin occurs via the sequential activation and conjugation of ubiquitin to target proteins by a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and an E3 (7). The majority of E3s contain an RF and mediate the transfer of ubiquitin directly from the associated E2 to one or more lysines of the specific target protein. Thus, the E3 confers specificity to the process. The Cbl proteins normally exist in the cytosol in an inactive state where the catalytic RF is masked by the N-terminal TKB domain (8–11). Upon activation of the kinases, the Cbl proteins bind directly (via the TKB, refs. 12–14) or indirectly via adaptor-dependent mechanisms (15, 16) to phosphotyrosines on the RTK (Fig. 1A). Phosphorylation of a conserved tyrosine in the linker region that separates the TKB from the RF of the Cbl

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proteins by the activated RTK (or other TKs) results in a dramatic structural rearrangement of the Cbl protein in which the N-terminal TKB rotates approximately 180 degrees (8, 10, 17). This exposes the RF allowing increased E2 binding to Cbl proteins and markedly increased E3 activity of the Cbl proteins. This structural rearrangement also positions the E2 in closer proximity to the RTK, facilitating the transfer of ubiquitin from the E2 to lysines on the RTK (8, 10, 17). Thus, activation of the RTK serves both 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. The ubiquitinylated RTK traffics through the endocytic compartments to the lysosome where it is degraded (Fig. 1A; reviewed in ref. 18).

Although the E3 activity of the Cbl proteins has been most extensively studied, the Cbl proteins can also function as adaptor molecules that recruit signaling molecules to activated RTKs (4–6, 18). This results in a positive role in signaling.
example, several studies have shown that the Cbl protein serves
as an adaptor to recruit PI3K to activated RTKs with subsequent
activation of the PI3K/AKT pathway (Fig. 1B; refs. 19, 20).

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Cbl proteins as drivers of cancer
v-Cbl was originally identified as an oncogene, causing leuke-
emia in mice and transforming NIH3T3 cells (21). The v-Cbl
protein contains only the TK8 domain of Cbl and, when expressed
in cells, prevents RTK ubiquitination and downregulation most
likely by acting as a dominant-negative protein preventing the
recruitment of endogenous Cbl proteins to the RTK (22, 23).
Other transforming mutants of the murine Cbl protein have been
identified from chemically induced lymphomas (70Z Cbl and
p95 Cbl; refs. 24, 25). These mutant proteins have in-frame
deletions of part or all of the linker and RF domains thus losing
E3 activity. Interestingly, these deletions result from point muta-
tions which lead to mis-splicing of the Cbl mRNA.

Mice deficient in Cbl, Cbl-b, or Cbl-c do not develop leukemia
(26). In contrast, mice that have a knockin of an RF-mutant Cbl
develop myeloid leukemia (26). The absence of leukemia in Cbl
knockout mice and the development of leukemia in mice with a
Cbl RING finger mutant knockin can be explained by a dom-
inant-negative function of the mutant protein, whereby the
mutant Cbl protein binds to activated RTKs and prevents recruit-
ment of the wild-type Cbl or Cbl-b proteins to the RTK. Con-
sistent with this, mice deficient in both Cbl and Cbl-b in
hematopoietic stem cells develop early onset of myeloid leuke-
emia (27). However, the positive functions of Cbl proteins in
signaling based on the adaptor function of Cbl suggest that the
mutant proteins may have both loss of tumor suppressor func-
tion (i.e., the loss of the negative regulatory E3 function) and
gain of oncogene function (e.g., coupling the RTK to down-
stream signaling pathways such as PI3K). Consistent with this,
the transforming 70Z form of Cbl activates the EGFR in the
absence of ligand and enhances activity of the EGF-R and down-
stream signaling upon ligand stimulation (28).

Cbl mutations have been found in approximately 5% of a wide
variety of myeloid neoplasms, including leukemias, lym-
hod tumors, chronic myelomonocytic leukemia (CMMML), and
Juvenile myelomonocytic leukemia (JMML). The frequency
of Cbl mutations appears to be highest in JMML (31), CMMML
(32), and secondary acute myeloid leukemia (AML and sAML, re-
spectively), atypical chronic myelogenous leukemia (aCML), CML
in blast crisis, chronic myelomonocytic leukemia (CMMML), and
juvenile myelomonocytic leukemia (JMML; reviewed in ref. 29).
The majority of these mutations are missense mutations that
classify within the linker region and within the RF domain lead-
ing to disruption of E3 activity (reviewed in ref. 29). The linker
region of Cbl tyrosine (Y371 in Cbl), whose phosphorylation is required for
E3 activity (as described above), is frequently mutated in myeloid
neoplasms accounting for approximately 15% of all missense
mutations (29, 30). These Y371 mutations occur mostly in
patients with JMML and CMMML (30–34). Deletions of all or
portions of the Cbl exon containing the distal portion of the
linker region and the proximal portion of the RF have been
described (29, 30). As seen in the murine Cbl deletion mutants,
these deletions result from mis-splicing due to mutations, inser-
tions, or deletions in the splice donor and acceptor sites surround-
ing exon 8. Nonsense mutations, frame shift mutations, and
insertions within the linker and RF regions have been found as
well (29). The missense mutations of Cbl are usually homozygous
mutations (resulting from copy neutral loss of heterozygosity—
also known as uniparental disomy), whereas the deletions that
arise from splicing mutations are more commonly heterozygous
(31–41). Transformation assays in NIH 3T3 cells found that
deletions of the linker region were transforming while point
mutations in the linker or RF were not (42). In addition, one
group found that 70Z Cbl induces greater ligand-independent
proliferation and survival than the R420Q mutation (43). How-
ever, others found no difference in transformation efficiency
between 70Z Cbl and a variety of point mutants found in patients
(34). Thus, it is unclear why most missense mutations are homo-
ygous and the deletion mutations are heterozygous.

Mutations of Cbl-b and Cbl-c are uncommon in myeloid
neoplasms, and the mutations found have not been functionally
characterized (37, 39). A total of five mutations of Cbl-b (out of
~2,000 patients evaluated) that are either frame shift or missense
mutations within the RF domain have been reported in myeloid
neoplasms (31, 37, 39–41). Several cases of frame shift or poly-
morphisms in the RF domain of Cbl-c have been reported, but
Cbl-c expression is restricted to epithelial cells, so the signifi-
cance of these abnormalities is unclear (39, 44–46).

v-Cbl also caused B-cell lymphomas in mice, but mutation in
human lymphoid malignancies is rare. Sequencing of Cbl in more
than 500 lymphoid malignancies found five somatic mutations,
three of which represent splice site mutations resulting in the loss
of a portion of the RF (30, 33, 47, 48). The Cancer Genome Atlas
(TCGA) sequencing programs have identified copy-number varia-
tions and mutations in solid tumors of the three Cbl genes in 0.3%
to 19.6% of the tumors, but the significance of these aberrations
is unknown (the results included here are in whole or part based
upon data generated by the TCGA Research Network: http://
cancergenome.nih.gov; ref. 49). Somatic mutations of Cbl have
been found in 10 non–small cell lung tumors out of 452 samples
(30, 50). All but one of the mutations described are outside the
linker and RING finger, and all are heterozygous. For those mutants
analyzed, E3 activity was maintained, but overexpression of these
mutants in lung cancer cells resulted in increased viability and
motility (50). This suggests that they may impair the association
of Cbl with a critical substrate, but the mechanism by which these
mutants affected viability or motility is unknown.

Therapeutic approaches to myeloid neoplasms containing
mutant Cbl proteins will require targeting the activated pathways
because the mutations in Cbl are at least partly loss-of-function
mutations. Activating mutations in Flt3 are found in approxi-
ately 30% of patients with AML, and inhibitors of Flt3 are being tested
for treatment of Flt3-mutant AML (51). Cbl ubiquitinates and
mediates lysosomal degradation of activated Flt3 (41). The devel-
opment of leukemia in Cbl RF mutant knockin mice is dependent
on Flt3 activity as crossing these mice to Flt3-deficient mice
abrogates the development of leukemia (26). Treatment of the
Cbl RF mutant knockin mice which had developed a myelopro-
liferative disorder with the high potency Flt3 inhibitor quinazinib
(AC220) significantly reduced the white blood count (73% reduc-
tion, P < 0.01), the spleen weight (69% reduction, P = 0.037), and
infiltration of the liver and lungs by myeloid cells (52). Treatment
with quinazinib induced quiescence in Flt3-dependent multipot-
ent progenitor cells (52). This suggests that myeloid neoplasms
containing a Cbl mutation are driven by Flt3 RTK and that
inhibiting this pathway may have efficacy for the treatment of
these neoplasms (26, 41). However, in the animal studies, the effect of Flt3 inhibition by quizartinib was not maintained once the drug was discontinued so that long-term treatment or combination therapy may be required (52). Alternatively, studies of the effects of mutant Cbl proteins on signaling have found enhanced activation of the PI3K/AKT and STAT5 pathways in the absence and presence of ligand (Fig. 1B; refs. 33, 34, 41). Importantly, the ligand-independent growth was inhibited approximately 70% to 80% by PI3K and mTOR inhibitors (33). Thus, targeting the PI3K pathway also is worth exploring in Cbl-mutant myeloid neoplasms.

As described above, although the E3 function of Cbl is lost due to mutations in cancers, the positive adaptor function is frequently maintained. Data suggest that the positive function contributes to the transforming potential of the Cbl mutants (ref. 34 and reviewed in ref. 29). A novel approach to targeting Cbl in myeloid neoplasms is to block the adaptor function of the Cbl protein to prevent activation of the downstream signaling pathway. The Cbl proteins bind to the activated RTKs via their TKB domain, and this allows recruitment of signaling proteins bound to other domains of Cbl to the RTK (e.g., PI3K kinase; refs. 19, 20). Thus, inhibition of the interaction of the Cbl TKB with the activated RTK would prevent the recruitment of signaling proteins to the activated RTK. On the basis of this, Kumar and colleagues (53, 54) are developing strategies to identify small molecules or peptides that bind the Cbl TKB and block interaction with the RTK. The efficacy of such an approach remains to be tested.

Cbl-b and adaptive and innate immune system

The loss of Cbl-b is associated with hyperactive T-cell immunity resulting in spontaneous and induced autoimmunity (55, 56). T cells from mice lacking Cbl-b have excessive proliferation and production of the cytokine IL2 that is uncoupled from the cells from mice lacking Cbl-b have excessive proliferation and production of the cytokine IL2 that is uncoupled from the CD28 costimulatory pathway (59, 60). Rather than leading to degradation, this loss of Cbl-b results in the production of the cytokine IL2 that is uncoupled from the CD28 costimulatory pathway (59, 60). Rather than leading to degradation, this loss of Cbl-b results in the production of IL2 which can lead to malignancy and/or to immune dysfunction. As we gain more knowledge of the signaling pathways affected in each case, novel therapeutic opportunities are arising for the treatment of cancer.

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Cbl Proteins and Cancer

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