Molecular Pathways

Deregulated Intracellular Signaling by Mutated c-CBL in Myeloid Neoplasms

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Abstract

c-CBL encodes a 120 kDa protein involved in intracellular signal transduction in a wide variety of cell types. Recently, frequent mutations of c-CBL have been reported in myeloid neoplasms showing both myelodysplastic and myeloproliferative features, where most mutations present in a homozygous state as a result of allelic conversion in 11q. c-CBL has a ubiquitin E3 ligase activity for a wide variety of tyrosine kinases, and thereby, negatively regulates tyrosine kinase signaling. In accordance with this, c-CBL seems to have tumor suppressor functions, loss of which promotes tumorigenesis. On the other hand, once mutated, it is converted to an oncogenic protein and commits to myeloid leukemogenesis through a kind of gain-of-function causing aberrant signal transduction. Inhibition of mutant CBL protein or signaling pathways it activates would have a role in therapeutics of myeloid neoplasms with CBL mutations.
Background

*c-CBL* proto-oncogene is a cellular counterpart of a viral oncogene, *v-CBL*, isolated from a transforming retrovirus that causes B-cell lymphoma and myeloid neoplasms in mice (1). *c-CBL* is recognized as a 120 kDa cytoplasmic protein rapidly phosphorylated after cytokine stimulation. Interacting with a broad spectrum of signaling and cytoskeletal molecules as a multi-adaptor protein as well as an E3 ubiquitin ligase, *c-CBL* is thought to be involved in intracellular signaling (2, 3). Although *c-CBL* was first identified through its oncogenic versions in mice, its role in human carcinogenesis has been elusive until recently, when frequent mutations of *c-CBL* have been reported in a subset of myeloid neoplasms (4-10). Mutations of *c-CBL* are found in a variety of myeloid neoplasms, including acute myeloid leukemia and myelodysplastic syndromes (4-7, 10, 11), but they are most frequent in those subtypes of myeloid neoplasms that are now grouped into myelodysplastic/myeloproliferative neoplasms (MDS/MPN) in the World Health Organization Classification (12). MDS/MPN include chronic myelomonocytic leukemia (CMML), juvenile myelomonocytic leukemia (JMML), and atypical chronic myeloid leukemia (aCML), where *c-CBL* mutations are found in 5% of aCML (8) and up to 15% JMML (9, 13, 14) and CMML (7, 10). They are originated from immature hematopoietic progenitors and commonly characterized by the production of dysplastic blood cells and myeloproliferative features (12, 15). Mutations seem to be somatic in most adult cases, but germline mutations were reported in some JMML cases in children (9). A conspicuous genetic feature of *c-CBL* mutations in these myeloid neoplasms is that mutations are homozygous in most cases as a result of an allelic conversion of 11q arms that leads to duplication of the mutated parental copy of 11q and loss of the remaining wild-type allele, i.e., “uniparental” disomy (UPD) of the 11q arms. Mutations rarely accompany deletions of the wild-type allele (7-10), indicating gain-of-function nature of the mutations rather than a simple loss-of-function (see below).

In mammals, three CBL homologues, c-CBL, CBL-b, and CBL-c, exist and are grouped into the CBL family of proteins (2, 3). All three homologues have a
conserved N-terminal domain (Tyrosine Kinase Binding (TKB) domain) for their binding to phosphorylated tyrosine kinases and a RING finger domain, as well as, an intervening linker sequence. c-CBL and CBL-b, but not CBL-c, have extended C-terminal structures, including a proline-rich domain, a ubiquitin-associated (UBA)/leucine zipper (LZ) motif at the C-terminus, and several tyrosine residues that are phosphorylated upon cytokine/growth factor stimulation (Figure 1). The TKB domain consists of a four-helix bundle (4H), a Ca^{2+}-binding EF hand (EF), and a variant Src homology 2 (SH2) domain (16), though which c-CBL binds to a phosphotyrosine-containing residue within a variety of activated tyrosine kinases. The spectrum of tyrosine kinases c-CBL can interact are thought to be determined by these N terminal structures and includes receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) (17-19), platelet-derived growth factor receptor (PDGFR) (20-22), insulin-receptor (23, 24), c-Kit (25, 26), and FLT3 (5, 27), as well as, non-receptor tyrosine kinases (JAK2, ZAP70, and Syk) (28). After targeted to activated tyrosine kinases, c-CBL initiates a series of interactions with a variety of molecules as a multi-adaptor protein to transmit signals (Figure 2a). First, c-CBL itself is phosphorylated at multiple tyrosine residues, to which a number of signaling molecules, including Vav (Y700) (29), Crk/CrkL (Y700 and Y774) (30-34), and the p85 subunit of PI3 kinase (Y731) (35, 36) are recruited. The proline-rich domain provides binding sites for a variety of Src homology 3 (SH3)-containing proteins, including Grb2 (17, 18, 26, 32, 37-39) and NCK (40, 41), Src family tyrosine kinases (Fyn and Src) (42-44), as well as, CAP and CIN85 (45, 46). Grb2 constitutively binds to c-CBL in unstimulated cells, playing a role in recruitment of c-CBL to phosphorylated RTKs when cells are stimulated with their ligands. Src family kinases are responsible for phosphorylation of c-CBL on RTK stimulation. The long list of molecules making direct or indirect interactions with c-CBL is found in an excellent review (3) and the list is still growing. The complexity of molecular interactions of CBL comprises ‘the CBL interactome’ and provides the basis for diverse biological functions of c-CBL. Among these, the most extensively studied are its function as a negative regulator of tyrosine kinase signaling.
The negative regulation of tyrosine kinases by c-CBL was first implicated through genetic studies in *C. elegans*, where the *c-CBL* orthologue, *sli-1*, was shown to be upstream of RAS (*let-60*) and Grb2 (*sem5*) and to suppress vulval induction that depends on *let23*, the orthologue of EGF receptor (47). Later, it was molecularly demonstrated in mammalian cells that the negative regulation involves multi-ubiquitinylation of RTKs (21, 48, 49). c-CBL has an E3 ubiquitin ligase activity, which is mediated by the linker/RING finger domains (50). c-CBL recruits E2 ubiquitin conjugating enzymes and ubiquitin monomers at the linker/RING finger interface and multi-ubiquitinylates the activated RTKs (Figure 2a, upper panel). Depending on the multi-ubiquitinylation of the kinases, the kinase-c-CBL complexes are directed to endocytosis for subsequent degradation at lysozomes and/or proteasomes, or for recycling (21, 48, 49), which in either case, limits kinase signals. Although multi-ubiquitinylation is critical for these reactions to occur, two c-CBL bound adaptor molecules, CIN85 and CD2AP, mediate the endocytosis (45, 46). The negative regulatory roles of c-CBL in tyrosine kinase signaling suggest that the protein could have an anti-oncogenic function. In fact, *c-CBL* null mice have an enlarged thymus, expanded hematopoietic progenitor pools, splenomegaly with extramedullary hematopoiesis, as well as increased repopulating capacity of their bone marrow cells (10, 51-53). Blastic transformation of chronic myelogeneous leukemia in a bcr/abl-transgenic model is accelerated in the *c-CBL* null background (10). Finally, *c-CBL* null mice developed invasive cancers with complete penetrance (unpublished data). Combined, these observations support that c-CBL can act as a tumor suppressor.

In contrast to the tumor suppressor function of the wild-type c-CBL, when transduced into NIH3T3 cells, *c-CBL* mutants isolated from human and murine neoplasms, as well as v-CEBL, show clear transforming capacity in terms of anchorage-independent growth in soft agar and tumor generation in nude mice (10, 54). Bone marrow cells transduced with mutant *c-CBL* (R420Q and 70Z mutants) generate generalized mastocytosis, a myeloproliferative disease, and myeloid leukemia in lethally irradiated mice with long latency but high penetrance (55). The transforming activity of mutant c-CBL seems to be mediated by alteration of the E3 ubiquitin ligase...
activity. Except for rare mutations causing a premature truncation of the TKB domain, most c-CBL mutations in myeloid neoplasms are missense changes at highly conserved amino-acid positions within the linker and RING finger domains or involve splice-site sequences, leading to amino acid deletions within these domains. While the E3 ubiquitin ligase activity primarily depends on the RING finger domain, the intact linker sequence, which tightly packs with the TKB domain as well as with the E2 ligase, is also considered to be essential for efficient ubiquitinylation to occur (56). The crystal structure of the c-CBL/UBCH7 complex suggests that Y371 is important for the integrity of the linker-TKB interface (56). Thus, tumor-derived c-CBL mutations are expected to affect the E3 ubiquitin ligase activity. In fact, linker-RING finger mutations found in myeloid neoplasms as well as other artificially introduced mutations within these domains were shown to have compromised E3 ubiquitin ligase activity (5, 8, 10, 54). Moreover, these c-CBL mutants strongly inhibit the E3 ligase activity of wild-type c-CBL, indicating that linker-RING finger mutants act in a dominant negative manner against wild-type c-CBL (10). This is expected because a simple loss-of-function would not explain the dominant effect of c-CBL mutant on transforming activity in NIH3T3 cells expressing wild-type c-CBL. Interestingly, this inhibitory effect does not seem to depend on dimerization with the wild-type c-CBL but on intact binding to phosphorylated tyrosine kinases, since a G306E mutation abolishes oncogenic capacity of these c-CBL mutants (unpublished data). Thus, when overexpressed in EGFR-transduced NIH3T3 cells, mutant c-CBL inhibits ubiquitinylation of EGFR, leading to prolonged activation of the receptor after EGF stimulation. Similarly, transduction of c-CBL mutants into hematopoietic cell lines results in prolonged activation of c-Kit, FLT3, and Jak2 kinases after stimulation with either their ligands or IL3 (10, 55) (Figure 2a, lower panel). Murine hematopoietic progenitors transduced with tumor-derived c-CBL mutants show increased cell survival in the presence of stem cell factor (SCF), similar to those from c-CBL null mice (10). Unexpectedly, however, the effect of these c-CBL mutants becomes much more prominent in the c-CBL null background, in which these c-CBL mutants induce exaggerated survival or even proliferative responses to SCF. Moreover, the augmented
proliferative/survival responses of mutant c-CBL-transduced cells are also found for a broader spectrum of cytokines, including thrombopoietin, IL-3, and FLT3 ligand (10). These effects of c-CBL mutants found in the c-CBL null background are not explained by either a simple loss of c-CBL functions or inhibition of wild-type c-CBL, but should be interpreted as true gain-of-function. Of particular interest, the gain-of-function of mutant c-CBL is lost in large part by the presence of either wild-type c-CBL allele or co-transduction of wild-type c-CBL. The gain-of-function becomes apparent in the c-CBL null background, explaining the observation that c-CBL mutations are found in a homozygous state with loss of the wild-type c-CBL in most cases (7-10).

Currently, the exact mechanism of the gain-of-function of c-CBL mutants is unclear. A possible mechanism is inhibition of CBL homologues (Figure 2b, red arrow) and/or CBL-intrinsic positive regulatory machinery (Figure 2b, blue arrow). Because the hypersensitive response to cytokines in mutant c-CBL-transduced cells is markedly diminished by wild-type c-CBL, it is mediated by inhibition of ‘CBL-like’ activity still present in c-CBL null cells, most likely CBL-b. Mutant c-CBL also inhibits E3 ubiquitin ligase activity of CBL-b, which is expressed in hematopoietic progenitor cells (10). c-CBL/CBL-b double knockout T-cells show exaggerated proliferative response to anti-CD3 stimulation and prolonged T-cell receptor signaling, as compared to c-CBL or CBL-b single knockout T cells (57). According to this model, two mutant c-CBL alleles could functionally titrate out two wild-type CBL-b alleles, while one mutant c-CBL allele might not be sufficient to overcome one wild-type c-CBL plus two wild-type CBL-b alleles (Figure 2c).

Another possible mechanism of the gain-of-function of mutated c-CBL is related to its function as a multi-adaptor, which is implicated in positive regulatory functions in signal transduction (Figure 2b, blue arrow). As an adaptor protein, kinase-bound c-CBL recruits a number of molecules involved in signal transductions and cytoskeletal regulations. For examples, upon either IL4 or G-CSF stimulation, c-CBL is tyrosine-phosphorylated and binds to the p85 subunit of PI3 kinase to transmit mitogenic/survival signals (58, 59). Similarly, CBL was shown to regulate integrin-mediated cell adhesion, spreading and migration in a PI3-kinase-dependent
manner (60, 61). It promotes activation of MAP kinases after stimulation of Met tyrosine kinase through binding to Crk (62). c-CBL is one of the downstream substrates/effectors of Src kinase signaling necessary for bone resorption and osteoclast migration (63). It is also involved in cytoskeletal regulation via activation of Rac1 or Cdc42, and R-RAS (64). In the face of loss of negative regulatory functions due to compromised E3 ubiquitin ligase activity, the intrinsic role in positive signaling of c-CBL protein could be unmasked as gain-of-function (Figure 2b). This model could explain the predominance of mutations in c-CBL but rare in CBL-b, because both proteins clearly have different functionalities as evident from different phenotypes of their knockout mice (51, 52, 65).

Clinical-Translational Advances

Gene mutations in signal transduction pathways are a common feature of myeloproliferative neoplasms. Deregulated kinase activity caused by bcr-abl and mutated JAK2 is a hallmark of chronic myelocytic leukemia and classical myeloproliferative disorders including polycythemia vera, essential thrombocytopenia, and primary myelofibrosis, respectively (66). Genes for receptor tyrosine kinases, such as platelet-derived growth factor receptors (PDGFRA/B) and fibroblast growth factor receptors (FGFR) are also recurrent targets of gene fusions in hyper eosinophilic syndrome (PDGFRA) and subsets of CMML (FGFR) (67). Finally, gene mutations commonly involving RAS pathway genes, including NF-1, RAS, and PTPN11, occurs in more than 70% of CMML cases, responsible for their hypersensitivity to GM-CSF (15, 67). The recent finding of frequent c-CBL mutations in the MDS/MPD subgroup revealed a novel mechanism for excessive cell signaling through deregulated kinase activity in myeloproliferative neoplasms, especially MDS/MPN subtypes, and also provided an insight into the therapeutics of c-CBL-mutated myeloid neoplasms.

Because c-CBL mutations induce excessive tyrosine kinase signaling, use of tyrosine kinase inhibitors could be a logical approach to the control of c-CBL-mutated neoplasms. However, the broad spectrum of c-CBL-regulated tyrosine kinases may preclude the efficacy of selective kinase inhibitors, whereas the use of pan-kinase...
inhibitors would increase a risk of the development of unacceptable adverse effects. Otherwise, identification of functionally relevant kinases regulated by mutated c-CBL would enable efficient targeting of such inhibition. Alternatively, the downstream signaling pathways, including JAK/STAT, PI3 kinase, as well as RAS/ERK signalings, are also potential therapeutic targets for inhibition with low molecular-weight (LMW) compounds.

Given that the gain-of-function nature of c-CBL mutants, inhibition of these mutant proteins would be a more reasonable approach, regardless of the exact mechanism of the gain-of-function. Since the oncogenic action of mutant c-CBL proteins depends on their intact binding to target kinases, inhibition of this binding would be a potential approach, especially when the inhibition could be specifically directed to mutant c-CBL but be saved for CBL-b. Recently, piceatannol, a naturally occurring phenol stilbenenoid was shown to induce loss of the CBL family of proteins including mutant CBL (70Z mutant) (68). Piceatannol was initially isolated as an antileukemic agent from a domesticated oilseed and was demonstrated to inhibit a broad spectrum of tyrosine kinases including Sky, Src, Lck, and FAK, as well as some serine/threonine kinases (69-72). It also induces selective loss of CBL-associated proteins; levels of PDGFRβ, c-Abl, and EGFR are reduced by piceatannol treatment, whereas those of c-Src, Lyn, Syk, and Grb2 are unaffected (68). The molecular mechanism that underlies piceatannol-induced CBL loss is still unclear. It does not depend on proteasome, lymosome, and caspase activation, but on reactive oxygen species, which seems to be distinct from the mechanism of inhibition of kinase activities (68). While piceatannol shows a broad spectrum of biological activity as anti-inflammatory, anti-histamine, and general anti-tumor agents in vitro (73-75), due to the broad biochemical actions of this agent, it has not been determined if or to what extent these biological activities of piceatannol depends on piceatannol-induced loss of CBL proteins. Although loss of both c-CBL and CBL-b is likely to result in increased tyrosine kinase activity, it also induces CBL-associated molecules and inhibits activity of a number of kinases, actually showing general anti-tumor activity. Unfortunately, no information is currently available as to the anti-tumor effect of piceatannol on c-CBL.
mutated leukemia. In c-CBL mutated leukemic cells, loss of mutant c-CBL may further augment anti-tumor activity of this agent.

Conclusion

c-CBL mutations are tightly associated with myeloproliferative myeloid neoplasms, especially the MDS/MPD subtype. c-CBL seems to act as a tumor suppressor, but when mutated, it is converted to an oncogenic protein. Although the oncogenic potential of c-CBL mutants is thought to be related to a type of gain-of-function, the molecular basis of this gain-of-function has not been fully understood. Undoubtedly, the effect of these mutations on the E3 ubiquitin ligase activity is essential for the gain-of-function. What complicates the mechanism is the fact that c-CBL has dual functionalities; it can behave as a multi-adaptor signal transducer, while also terminating signals by ubiquitinylating activated tyrosine kinases. Clearly, to understand the exact oncogenic mechanism of c-CBL mutants and to develop effective therapeutics, further in vivo and in vitro analyses are required.
Figure Legends

Figure 1.
Structure of CBL family proteins and c-CBL mutations
a, Domain structures of CBL family proteins are depicted. Major tyrosine phosphorylation sites in c-CBL are indicated. Molecular interactions of c-CBL with cytokine receptors and other signaling molecules are also shown on top.

Figure 2.
Putative mechanism of gain-of-function of c-CBL mutants
a, After cytokine (growth factor) stimulation, receptor tyrosine kinases (RTKs) are phosphorylated, to which c-CBL or CBL-b binds to ubiquitinate the receptors, while participates in signal transduction. Ubiquitinated RTKs are then subjected to degradation or recycling. On the other hand, when mutant c-CBL binds to the activated RTKs, downregulation of the RTKs is compromised, leading to prolonged signaling. b, Putative mechanisms of the gain-of-function of c-CBL mutants; the CBL-b-inhibition model (red line) and the mechanism mediated by positive regulatory functions of c-CBL (blue line). c, In the CBL-b-inhibition model, a c-CBL mutant inhibits the E3 ubiquitin ligase activity of both c-CBL and CBL-b. In the heterozygous state, the inhibitory action of the c-CBL mutant is largely titrated out by three intact copies of c-CBL and CBL-b, leading to only modest increase in sensitivity to cytokines, as compared with the normal state (middle panel). When the mutant allele is duplicated by an allelic conversion in 11q, the mutant protein expressed from the two mutated alleles can effectively inhibit the remaining enzymatic activity from CBL-b (right panel).
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