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, in which most mutations are present in a homozygous state, as a result of allelic conversion in 11q. c-CBL has ubiquitin E3 ligase activity for a wide variety of tyrosine kinases, and thereby, negatively regulates tyrosine kinase signaling. Accordingly, 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. The inhibition of mutant CBL protein or signaling pathways that it activates would have a role in therapeutics of myeloid neoplasms with CBL mutations.

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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 (CML), juvenile myelomonocytic leukemia (JMML), and atypical chronic myeloid leukemia (aCML). c-CBL mutations are found in 5% of aCML (8) and up to 15% of JMML (9, 13, 14) and CML (7, 10). They originate from immature hematopoietic progenitors and are 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, or “uniparental” disomy of the 11q arms. Mutations rarely accompany deletions of the wild-type allele (7–10), indicating the gain-of-function nature of the mutations rather than a simple loss-of-function (see below).

In mammals, three CBL homologs, c-CBL, CBL-b, and CBL-c, exist and are grouped into the CBL family of proteins (2, 3). All three homologs 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–leucine zipper motif at the C terminus, and several tyrosine residues that are phosphorylated upon cytokine and/or growth factor stimulation (Fig. 1). The TKB domain consists of a four-helix bundle, a Ca2+-binding EF hand, and a variant Src homology 2 (SH2) domain (16),...
through which c-CBL binds to a phosphotyrosine-containing residue within a variety of activated tyrosine kinases. The spectrum of tyrosine kinases with which c-CBL can interact is thought to be determined by these N-terminal structures and includes receptor tyrosine kinases (RTK), such as epidermal growth factor receptor (EGFR; refs. 17–19), platelet-derived growth factor receptor (PDGFR; refs. 20–22), insulin-receptor (23, 24), c-Kit (25, 26), and FLT3 (5, 27), as well as non-RTKs (JAK2, ZAP70, and Syk; ref. 28). After being 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 (Fig. 2A). First, c-CBL itself is phosphorylated at multiple tyrosine residues, to which a number of signaling molecules, including Vav (Y700; ref. 29), Crk/CrkL (Y700 and Y774; refs. 30–34), and the p85 subunit of PI3 kinase (Y731; refs. 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; refs. 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 the diverse biological functions of c-CBL. Among these, the most extensively studied is 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 Caenorhabditis elegans, in which the c-CBL ortholog, sli-1, was shown to be upstream of RAS (let-60) and Grb2 (sem5), and to suppress vulval induction that depends on let-23, the ortholog of EGFR (47). Later, it was molecularly shown in mammalian cells that the negative regulation involves multi-ubiquitinylation of RTKs (21, 48, 49). c-CBL has 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 (Fig. 2A, upper panel). Depending on the multi-ubiquitinylination of the kinases, the kinase-c-CBL complexes are directed to endocytosis for subsequent degradation at lysosomes 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 myelogenous 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.9 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-CBL, show clear transforming capacity in terms of

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9 Unpublished data.
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. Although 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 ubiquitinylolation 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 finding 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, because a G306E mutation abolishes oncogenic capacity of these c-CBL mutants.10 Thus, when overexpressed in EGFR-transduced NIH3T3 cells, mutant c-CBL inhibits ubiquitylation 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 interleukin 3 (IL-3; Fig. 2A, lower panel; refs. 10, 55). Murine hematopoietic progenitors transduced with tumor-derived c-CBL mutants show increased cell survival in the presence of stem cell factor, 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 stem cell factor. Moreover, the augmented proliferative and/or 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 on c-CBL is lost in large part by the presence of either wild-type c-CBL allele or cotransduction 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 homologs (Fig. 2B, red arrow) and/or CBL-intrinsic positive regulatory machinery (Fig. 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 with 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, whereas one mutant c-CBL allele might not be sufficient to overcome one wild-type c-CBL plus two wild-type CBL-b alleles (Fig. 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 (Fig. 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 IL-4 or granulocyte colony-stimulating factor stimulation, c-CBL is tyrosine-phosphorylated and binds to the p85 subunit of phosphoinositide 3 kinase (PI3K) to transmit mitogenic and/or survival signals (58, 59). Similarly, CBL was shown to regulate integrin-mediated cell adhesion, spreading, and migration in a PI3K-dependent manner (60, 61). CBL promotes activation of MAP kinases after stimulation of Met tyrosine kinase through binding to Crk (62). c-CBL is one of the downstream substrates and/or 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 (Fig. 2B). This model could explain the observation that c-CBL mutations were much more frequent than CBL-b mutations in MDS-MPN, because both proteins clearly have different functionalities, as evident from the different phenotypes of their knockout mice (51, 52, 65).

Clinical-Translational Advances

Gene mutations in signal transduction pathways are a common feature of MPN. 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 thrombocytemia, and primary myelofibrosis (66). Genes for RTKs, such as PDGFRs (PDGFRA/B) and fibroblast growth factor receptors (FGFR) are also recurrent targets of gene fusions in hypereosinophilic syndrome (PDGFRA) and subsets of CMML (FGFR; ref. 67). Finally, gene mutations commonly involving RAS pathway genes, including NF-1, RAS, and PTPN11, occur in more than 70% of CMML cases, responsible for their hypersensitivity to granulocyte-macrophage colony-stimulating factor (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 MPN, 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

10 Unpublished data.
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 the 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, PI3K, as well as RAS/extracellular signal-regulated kinase (ERK) signalings, are also potential therapeutic targets for inhibition with low molecular-weight compounds.

Given 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. Because 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; ref. 68). Piceatannol was initially isolated as an antileukemic agent from a domesticated oilseed and was shown 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 protesome, lysosome, and caspase activation, but rather on reactive oxygen species, which seems to be distinct from the mechanism of inhibition of kinase activities (68). Although piceatannol shows a broad spectrum of biological activity as an anti-inflammatory, antihistamine, and general antitumor agent in vitro (73–75), because of its broad biochemical actions, it has not been determined if, or to what extent, the biological activities of piceatannol depend 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 antitumor activity. Unfortunately, no information is currently available about the antitumor effect of piceatannol on c-CBL-mutated leukemia. In c-CBL-mutated leukemic cells, loss of mutant c-CBL may further augment antitumor 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 compromises 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 ubiquitiny-lating 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.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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