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Peptide truncation leads to a twist and an unusual increase in affinity for casitas B-lineage lymphoma tyrosine kinase binding domain

Eric A. Kumar^{†,‡}, Ziyang Yuan^{†,‡}, Nicholas Y. Palermo[†], Lin Dong[†], Gulzar Ahmad[†], G.L. Lokesh[‡], Carol Kolar[†], Smitha Kizhake^{†,‡}, Gloria E. O. Borgstahl^{†,‡}, Hamid Band^{†,§,||,o,a,b}, and Amarnath Natarajan^{†,‡,§,‡,*}

[†]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

[#]Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

[§]Department of Genetics, Cell Biology and Anatomy, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

^{||}Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

^oDepartment of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

^aDepartment of Pharmacology and Experimental Neuroscience, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

^bCollege of Medicine, University of Nebraska Medical Center, Omaha, Nebraska, 68022 United States

[‡]Chemical Biology Program, Department of Pharmacology, University of Texas Medical Branch, Galveston, Texas, 77555 United States

Abstract

We describe truncation and SAR studies to identify a pentapeptide that binds Cbl tyrosine kinase binding domain with a higher affinity than the parental peptide. The pentapeptide has an alternate binding mode that allows occupancy of a previously uncharacterized groove. A peptide library was used to map the binding site and define the interface landscape. Our results suggest that the pentapeptide is an ideal starting point for the development of inhibitors against Cbl driven diseases.

Keywords

Cbl inhibitors; protein-peptide complex; in silico screen; protein-protein interactions

The Casitas B-Lineage Lymphoma (Cbl) family of proteins plays a fundamental role in controlling tyrosine kinase-coupled cellular signaling pathways. A family of multi-domain

*Corresponding Author: Phone: (402) 559-3793, anatarajan@unmc.edu.

Supporting information. experimental methods, tables S1–S4, and figures S1–S6 included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

proteins that functions as scaffold-type E3 ubiquitin ligases, Cbl proteins specialize in negatively regulating activated protein tyrosine kinases (PTKs).^{1–5} The Cbl-PTK interaction is a phosphorylation driven event that involves direct interaction between the N-terminal tyrosine kinase binding domain (TKB) of Cbl with cognate phosphotyrosyl peptide motifs on PTKs.^{6, 7} The E3 ubiquitin ligase activity is mediated by the RING finger domain adjacent to the TKB domain. A highly conserved linker region bridges the TKB and the RING domains, and participates with the RING finger in physical interactions with E2 ubiquitin conjugating enzymes (UBCs).⁸ Within the C-terminal half, a proline rich region (PRR) mediates binding of SH3 domain-containing proteins, while induced tyrosine phosphorylation sites allow complexes with SH2 domain-containing proteins. These interactions underscore the scaffolding function of the C-terminal sequences in juxtaposing additional targets to Cbl.⁹ Targets recruited to Cbl by the TKB domain or the C-terminal interactions are subjected to ubiquitination and negative regulation. From a biochemical standpoint, inhibitors specific to Cbl(TKB) could serve to not only prevent Cbl recruitment to PTKs but also to decouple the PTK binding function from the C-terminal region-mediated scaffolding function.

Several groups have recently identified oncogenic Cbl mutations within the RING and linker regions that are associated with a subset of human myelomonocytic leukemias.^{10–14} The mutant Cbl allele is typically duplicated in a copy number-neutral loss of heterozygosity referred to as acquired uniparental disomy (aUPD), which has suggested a potential gain-of-oncogenic function of oncogenic Cbl mutants.^{15, 16} Clinically-relevant Cbl mutations, where analyzed, result in the loss of E3 ligase function but with maintenance of the protein-protein interactions suggesting that E3-deficient oncogenic mutants acquire a scaffolding function to assemble abnormal signaling complexes to promote sustained PTK signaling.^{17–20} Since Cbl(TKB) interacts with a large number of PTKs, targeting these enzymes for therapy of cancers driven by Cbl mutations is likely to be limited. On the other hand, a more elegant approach would be to target the mutant Cbl proteins themselves.^{21, 22} Data from our lab and others suggests that the transforming capacity of Cbl mutants depends on PTK binding, inhibitors of the Cbl(TKB)-PTK interaction could provide a novel means to treat leukemias driven by Cbl(linker/RING) mutations.^{21, 22, 22} The ability to reversibly disrupt the Cbl(TKB)-PTK interaction could also serve as a chemical biological approach to disrupt the function of Cbl proteins and to boost physiological responses in which Cbl proteins play a negative regulatory role.

The systematic development of small molecule inhibitors (SMIs) that perturb protein-protein interactions is becoming more feasible for generating biochemical reagents and therapeutics. From a biochemical standpoint, these compounds can serve as valuable tools to dissect the complex signaling networks in cellular systems. SMIs enjoy several advantages over classical biochemical techniques (i.e., gene knockouts, silencing RNA, mutagenesis). The rapid nature of small molecule inhibition permits the investigation of interactions that occur at much faster time scales; a necessary feature when the real-time responses of cellular pathways are being considered. The tunable nature allows for titrated inhibition, as opposed to the simple on/off mechanism afforded by genetic methods. The reversible nature of small molecule inhibition allows one to disrupt an interaction, observe the response, and end the disruption to recapture the initial condition. Taken together, these attributes offer the ability to directly perturb cellular pathways at the protein level without altering the genetic background of the system. SMIs, therefore, can provide the resolution needed to describe the role of a domain-specific, protein-protein interaction within a given pathway.

In view of the lack of Cbl(TKB) inhibitors and given their biological and clinical relevance, we undertook a peptidomimetic approach. The initial steps in the peptidomimetic design process include: (1) the identification of a natural ligand, (2) defining the minimal active

sequence (3) and the identification of the key residues and their relative 3D arrangement required for activity.^{23, 24} In this report, we used a combination of methods to identify an optimal Cbl(TKB)-binding lead sequence suitable for structure-guided design. We report an unusual increase in the binding affinity through truncation of a 12-mer Cbl(TKB) binding peptide. We predicted that the pentapeptide (**1**, pYTPEP) and its parental 12-mer peptide (**2**, TLNSDGpYTPEPA) will have similar binding affinity for Cbl. Interestingly, the biochemical data showed that **1** had ~3–8-fold higher affinity for Cbl(TKB) compared to **2**. Computational studies with **1** suggest that the N-terminus of the pentapeptide rotates to occupy a shallow groove adjacent to the previously defined binding site. Such an interaction is not feasible with the 12-mer peptide **2**. We propose that this flexibility and reorientation is the source of the improved affinity of **1** compared to **2**. In addition, a library of pentapeptides was used to map the binding interface and define the optimal conformation of the ligand. (See Table S1 for numbering of peptides)

Based on the available crystal structures (Figure 1), we generated a truncation peptide set by sequentially removing amino acids from each terminus.^{25–27} We started with the 9-mer peptide sequences as this was the longest sequence in which each residue could be assigned 3D coordinates based on the crystal structures. The truncation set was screened *in silico* against Cbl(TKB) to identify the smallest unit that retained binding affinity (Table S2). The binding energies were calculated using AutoDock Vina in single calculation mode.²⁸ Unexpectedly, truncating the peptide to include only the pYTPEP sequence (**1**) resulted in a slight increase in binding affinity. Based on this finding, we predicted that the truncated pentapeptide would exhibit comparable binding affinity and provide a reasonable lead compound for a peptidomimetic inhibitor design strategy (Figure S1).

To test this hypothesis, we used a set of orthogonal techniques, *viz.* fluorescence polarization (FP), isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) (see supporting information Table S3 and Figure S2–S3) and TKB domain pull-down experiments. We recently reported the development and miniaturization of a Cbl(TKB) FP assay for high throughput screening to identify Cbl(TKB) inhibitors.²⁹ Here, the FP assay was adapted to quantify binding of 12-mer peptide or pentapeptide to Cbl(TKB) and inhibition of binding by various peptides. Peptides **1** and **2** were labeled with a fluorophore at the N-terminus to generate two probes (**3**, Flu-pYTPEP and **4**, Flu-TLNSDGpYTPEPA) for the FP assay. In two separate experiments, Cbl(TKB) was titrated into a constant concentration of each probe. The K_d values were found to be $2.4 \pm 0.1 \mu\text{M}$ for **3** and $6.2 \pm 0.3 \mu\text{M}$ for **4** (Table 1). These direct binding experiments demonstrated that **3** bound with ~3-fold better affinity than **4**.

It is feasible that the fluorescent label at the N-terminus of **3** could replace the extended residue sequence of **4** and contribute to the binding affinity. To rule out this possibility, a set of competitive binding experiments were performed where each peptide was titrated vs. a constant concentration of Cbl(TKB) and the fluorescently-labeled probe. Titration of **1** and **2** vs. **3** showed that the unlabeled pentapeptide bound with ~6-fold better affinity (Table 1). Similarly, titration of **1** and **2** vs. **4** showed that the unlabeled pentapeptide bound with ~8-fold better affinity. As expected, the unlabeled peptides were able to competitively compete for binding with the matched labeled probe and with the unmatched labeled probe. The ability of the pentapeptide to displace the 12-mer probe and *visa versa* supports the hypothesis that **1** occupies the same binding site as **2**. Moreover, the K_i values were comparable when compared across each probe. That is, the change in probe affinity is accounted for when determining the binding constant for the inhibitor (K_i) in a competitive binding experiment.

Next, ITC, was used as an orthogonal technique to directly determine Cbl(TKB)-peptide binding. In separate experiments, **1** and **2** were titrated against a solution of Cbl(TKB) and the heat changes of the solution upon binding were measured. These direct binding experiments demonstrated that the short peptide binds Cbl(TKB) with up to ~5-fold improvement in the K_a when compared to the long peptide (Table 1). These results are consistent with those observed from the FP measurements.

To determine if the higher affinity of pentapeptide **1** vs. the 12-mer **2** can translate into better inhibition of the Cbl(TKB) interaction with an intact cellular binding partner, we conducted pull-down experiments with activated Epidermal Growth Factor Receptor (EGFR) expressed in an immortal human mammary epithelial cell line 16A5.³¹ In a typical experiment, cells were stimulated with EGF, which results in the phosphorylation of EGFR (pEGFR). GST- Cbl(TKB) fusion protein isolated on glutathione-Sepharose beads was used to pull-down activated EGFR from lysates of EGF-stimulated cells in the absence or presence of increasing concentrations of each peptide. After 1 hr incubation, the beads were spun down, and the bound pEGFR protein was visualized using Western blotting for pTyr. The results from the pull-down experiments are summarized in Figure 2. As expected, we observed a decrease in the binding of pEGFR with increasing concentrations of the inhibitor peptides. Moreover, **1** has ~3-fold better IC_{50} value compared to **2**. Together, the biochemical studies clearly show that the computationally predicted pentapeptide **1** binds Cbl(TKB) with ~3–8-fold higher affinity compared to the 12-mer peptide **2**.

To identify the key residues required for binding within the pentapeptide **1**, an alanine scan was performed in which each residue was sequentially replaced with an Ala (**15–20**) residue. We, and others, have shown that the phosphotyrosine is a key binding component as the non-phosphorylated Tyr-containing peptide does not bind Cbl(TKB).^{6, 7, 29} Analysis of the 33G values relative to **1** reveals that each residue modestly contributes to Cbl(TKB) binding (Figure 3A, cyan bars). Interestingly, there is a significant loss of activity in **19**, suggesting that the proline residue at the P+4 position makes substantial contribution to binding. Introduction of a carboxylic acid in place of carboxamide on the C-terminal P+4 proline also results in the loss of activity in **20**, which highlights the hydrophobic nature of the interaction. Taken together, this indicates that **1** is anchored to Cbl(TKB) in a two-point binding fashion. These sites can potentially be exploited to improve inhibition. Furthermore, removal of the acyl group at the N-terminus, in **15**, results in a loss of ΔG of ~2 kcal·mol⁻¹. This change suggested that there is a possible third anchoring interaction.

Several 12-mer peptides have been identified that bind Cbl(TKB) and the crystal structures of many of these complexes have been solved.^{25, 26} Based on these reports, and our observations, each of the known Cbl(TKB)-binding peptides was truncated to the corresponding pentapeptide sequences and tested for binding-inhibitory activity (Figure 3A, white, grey, and black bars). It was observed that those peptides that adopt the same binding mode and orientation as **1** have comparable affinity (**21**, Figure 3C). The peptide orientations that deviate slightly lose activity by ~1 order of magnitude (**22–24**, Figure 3D) and the peptide backbones that deviate significantly from **1** have ~2 orders of magnitude lower activity (**25–26**, Figure 3E). These results suggest that, in addition to the contacts made by the phosphate group and the P+4 Pro residue, the backbone geometry might contribute to Cbl(TKB) binding.

In parallel, we conducted a computational study to determine binding affinities of the peptides in the library depicted in Figure 3. A plot of the experimentally-determined vs. the computed binding affinities resulted in an R^2 value of 0.68 (Figure 4A). This suggests that, at least for this system, a docking-guided peptidomimetic inhibitor optimization will be reliable.

Based on the fidelity of our computational studies, we were able to determine the most active binding mode of **1** and overlaid the docked structure onto the crystal structure of **2** (Figure 4B and Table S4). The ΔG_{Calc} of the best conformation was $-8.2 \text{ kcal}\cdot\text{mol}^{-1}$. It was observed that when the residues N terminal to the pTyr are removed, the free acyl group can rotate to occupy a shallow pocket situated adjacent to the pTyr binding site (Figure 1). The pocket is composed of residues Arg₂₉₉, Gln₃₀₂, Ile₃₁₈, and His₃₂₀ (Figure S1). Based on these observations, it seems likely that the N-terminal acyl group of pentapeptide **1** forms a hydrogen bond with the Arg₂₉₉ guanidinium group. The Arg₂₉₉ side chain is at prime interacting distance of 2.82 Å and oriented in the optimum collinear geometry (Figure S1). This observation is consistent with the resulting loss in affinity when the acyl group is removed (Figure 3A; peptide **15**). To test this prediction, the oxygen was replaced with a carbon to disrupt hydrogen bond formation, yielding peptide **27** (Figure S1). The ΔG_{Calc} was found to be $-7.6 \text{ kcal}\cdot\text{mol}^{-1}$ ($\Delta\Delta G = 0.6 \text{ kcal}\cdot\text{mol}^{-1}$). **2** results in a loss of $\sim 1 \text{ kcal}\cdot\text{mol}^{-1}$ in binding affinity and is sterically inhibited from occupying the shallow pocket. Together our data suggest that the pocket formed by the residues Arg₂₉₉, Gln₃₀₂, Ile₃₁₈ and His₃₂₀ can be exploited to generate inhibitors with higher affinity. This is currently under investigation.

In conclusion, we have used tandem computational and experimental approaches to identify the minimal peptide unit capable of binding Cbl(TKB). The computational studies predicted that a pentapeptide (**1**) is sufficient for binding and indicated that it is likely a two-point binding mode. Cell-free assays confirmed the interaction of **1** with Cbl(TKB) in both direct binding and competitive inhibition assays. Further, these studies demonstrated that pentapeptide **1** bound with a higher affinity than the 12-mer peptide **2**. A pull-down assay with full length proteins revealed that these peptides could inhibit the Cbl(TKB)-protein interaction and that **1** has ~ 3 -fold better activity than **2**, which is consistent with the cell-free binding assays. A SAR study with a small library of pentapeptides indicated that **1** is the minimal unit and that the backbone geometry is important. In parallel, results from a docking study correlated well with the experimental data for the peptide library. Finally, the predicted binding mode of **1** revealed a third point of interaction adjacent to the binding site of the pTyr residue that can be occupied in the absence of the extended N-terminus of **2**. The additional H-bond and buried surface could contribute to the observed increase in binding affinity of the pentapeptide compared to the 12-mer peptide. We are currently exploiting these observations to develop high affinity Cbl(TKB) inhibitors that will be reported in due course of time.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Cbl(TKB)	casitas B-lineage lymphoma (Tyrosine Kinase Binding Domain)
PTK	protein tyrosine kinase

FP	fluorescence polarization
PPI	protein-protein interaction
ITC	isothermal titration calorimetry
SAR	structure activity relationship
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
pEGFR	phospho-epidermal growth factor receptor

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Figure 1.

Graphic illustrating the binding mode of the 12-mer peptide (black cartoon) in complex with Cbl(TKB) (blue surface). The yellow surface represents the pTyr binding site and the white surface represents the Pro5 binding site. The red surface represents an adjacent binding site. (PDB ID: 1FBV).

Figure 2.

Differential competition of pEGFR pull-down by GST-Cbl(TKB) in the presence of increasing amounts of (A) **2** or (B) **1** assessed using immunoblotting for pEGFR with an anti-phosphotyrosine antibody 4G10. Negative control peptides do not inhibit the Cbl(TKB) pull-down of pEGFR. Band intensities were quantified using Image-J software and pull-down IC_{50} values were determined by nonlinear regression using SigmaPlot.

Figure 3.

(A) $\Delta\Delta G$ values of a focused peptide library of truncated Cbl(TKB) binding peptides. The reported $\Delta\Delta G$ is relative to **1**. K_i was calculated according to the Coleska-Wang equation and $\Delta G = RT \ln K_i$.³⁰ (The reported values were determined from 3 separate FP experiments). (B–E) Overlaid crystal structures of Cbl-binding peptides illustrating the backbone geometry relative to (B) the highest affinity peptide (**1**, blue). (C) **21** (brown), (D) **23** (green), (E) **25** (orange).

Figure 4.

(A) Correlation of binding affinities based on experimental data (ΔG_{Exp}) and docking studies (ΔG_{Calc}). Solid lines represent the best linear fit and the dashed lines represent the 95% confidence interval. (B) Calculated binding mode of **1** (green sticks) and crystal structure of **2** (black sticks) in complex with Cbl(TKB) (color coordinated as Figure 1). The coordinates for the truncated peptide was generated using a hybrid docking molecular dynamics simulation and the graphic was generated using PyMOL.³² (PDB ID: 1FBV)

Table 1

(FP) Direct- and competitive-binding experiments with the 12-mer and pentapeptides. The K_i for competitive inhibition was calculated by the Coleska-Wang equation (K_d and $K_i \pm \text{SEM}$).³⁰ (ITC) Thermodynamic components of Cbl(TKB)-peptide binding. ΔG was calculated by the equation: $\Delta G = -RT \ln K_a$. The parameters reported are from two separate experiments.

Fluorescence Polarization (FP)			
Probe	Inhibitor Peptide	K_d (μM)	K_i (μM)
Flu-Short (3)	-	2.4 ± 0.1	-
Flu-Long (4)	-	6.2 ± 0.3	-
Flu-Short (3)	1	-	0.8 ± 0.1
Flu-Short (3)	2	-	4.6 ± 0.4
Flu-Long (4)	1	-	0.7 ± 0.3
Flu-Long (4)	2	-	5.5 ± 0.8
Isothermal Titration Calorimetry (ITC)			
Peptide	N	K_a ($\times 10^5$) (M^{-1})	ΔG_{ITC} ($\text{kcal}\cdot\text{mol}^{-1}$)
1	0.93 ± 0.03	14.3 ± 1.6	-8.38 ± 0.03
2	0.93 ± 0.07	2.9 ± 0.5	-7.46 ± 0.04