The Cbl Phosphotyrosine-binding Domain Selects a D(N/D)XpY Motif and Binds to the Tyr\(^{292}\) Negative Regulatory Phosphorylation Site of ZAP-70*  

(Received for publication, June 20, 1997, and in revised form, November 6, 1997)

Mark L. Lupher, Jr.,§§, Zhou Songyang¶, Steven E. Shoelson¶, Lewis C. Cantley¶, and Hamid Bandi**  

From the Lymphocyte Biology Section, Division of Rheumatology, Immunology and Allergy, Department of Medicine, Brigham and Women's Hospital, the Division of Signal Transduction, Beth Israel Deaconess Hospital Medical Center, and the Research Division, Joslin Diabetes Center, Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115

The Cbl protooncogene product has emerged as a novel negative regulator of receptor and non-receptor tyrosine kinases through currently undefined mechanisms. Therefore, determining how Cbl physically interacts with tyrosine kinases is of substantial interest. We recently identified a phosphotyrosine binding (PTB) domain residing within the N-terminal transforming region of Cbl (Cbl-N), which mediated direct binding to ZAP-70 tyrosine kinase. Here, we have screened a degenerate phosphopeptide library and show that the Cbl-PTB domain selects a D(N/D)XpY motif, reminiscent of but distinct from the NPXpY motif recognized by the PTB domains of Shc and IRS-1/2. A phosphopeptide predicted by this motif and corresponding to the in vivo negative regulatory phosphorylation site of ZAP-70 (Tyr\(^{(P)^{292}}\)) specifically inhibited binding of ZAP-70 to Cbl-N. A ZAP-70/Y292F mutant failed to bind to Cbl-N, whereas a D290A mutant resulted in a 64% decrease in binding, confirming the importance of the Tyr\(^{(P)}\) and Y-2 residues in Cbl-PTB domain recognition. Finally the ZAP-70/Y292F mutant also failed to associate with Cbl-N or full-length Cbl in vivo. These results identify a potential Cbl-PTB domain-dependent role for Cbl in the negative regulation of ZAP-70 and predict potential Cbl-PTB domain binding sites on other protein tyrosine kinases known to interact with Cbl.

We and others have previously demonstrated that p120\(^{cd} \) (Cbl), the product of the c-cbl proto-oncogene, is a target of tyrosine phosphorylation upon stimulation through a number of cell surface receptors coupled to tyrosine kinase activation (reviewed in Refs. 1–3). To characterize the function of Cbl and the structural basis for its function, we have investigated its role in the T cell antigen receptor (TCR)\(^3\) signaling cascade. Cbl is one of the earliest proteins to be tyrosine phosphorylated upon TCR stimulation (1–3), and recent work has demonstrated that Cbl is a substrate for ZAP-70 and Syk kinases and associates with ZAP-70 and Syk in vivo (4–9). We previously demonstrated that the N-terminal transforming region of Cbl (Cbl-N) contains a phosphotyrosine binding (PTB) domain that directly binds to phosphorylated ZAP-70 in activated T cells (13). A point mutation within Cbl-N, corresponding to a loss-of-function mutation in SLI-1, the Caenorhabditis elegans Cbl homologue, abrogated Cbl-N binding to ZAP-70. SLI-1 was identified as a negative regulator of the LET-23 receptor tyrosine kinase (a homologue of the mammalian EGF receptor (EGFR)) (11), and a similar role has been deduced for the Drosophila homologue of Cbl (12). In addition, overexpression of Cbl in mammalian cells was also shown to reduce the tyrosine kinase activity of Syk, suggesting that mammalian Cbl may also be a negative regulator of nonreceptor tyrosine kinases (7). Furthermore, utilizing dominant oncogenic mutants of Cbl, which induce an up-regulation of the PDGFR\(\alpha\) signaling cascade in transfected NIH3T3 fibroblasts, we have now demonstrated a requirement for the Cbl-PTB domain in tyrosine kinase regulation (14). Together these data indicated that PTB domain-mediated interactions may be critical for the tyrosine kinase regulatory function of Cbl. Therefore, identification of Cbl-PTB domain-binding sites on its target tyrosine kinases, such as ZAP-70, is likely to elucidate an important aspect of tyrosine kinase regulation.

In this study, we have used screening of a phosphopeptide library to identify the Cbl-PTB domain-binding motif. Based on the determined D(N/D)XpY motif, we identify the binding site for the Cbl-PTB domain in ZAP-70 to be the in vivo negative regulatory site Y292 (45, 46). Using mutagenesis of ZAP-70, we confirm that both the phosphotyrosine (Tyr\(^{(P)^{292}}\)) and the Asp at the Y-2 position (Asp\(^{295}\)) are important for recognition by the Cbl-PTB domain and show that Tyr\(^{292}\) is required for in vivo association of ZAP-70 with full-length Cbl. These data identify Cbl as a potential negative regulator of ZAP-70 and support a model whereby Cbl may act as a general negative regulator of tyrosine kinases through a Cbl-PTB domain-dependent mechanism. In addition, our results allow prediction of possible binding sites for the Cbl-PTB domain on other tyrosine kinases known to interact with Cbl.

** Experimental Procedures  

Cells and Activation—Jurkat cells (clone E6.1; ATCC), were maintained, activated, and lysed as described (13). COS-7 cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum; EGF, epidermal growth factor; EGFR, EGF receptor; PTB, phosphotyrosine-binding domain; Cbl-N, N-terminal transforming region of Cbl; HA, hemagglutinin; PDGFR\(\alpha\), platelet-derived growth factor \(\alpha\) receptor.
10% fetal calf serum (HyClone), 20 mM HEPES, pH 7.35, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Life Technologies, Inc.).

Antibodies—The antibodies used in this work were as follows: 4G10 (anti-Tyr(P), IgG2a) (15), SPV-T3b (anti-CD3e; IgG2a) (16), anti-ZAP-70 (IgG2a; Z24820, Transduction Laboratories, Lexington, KY), 9E10 (anti-myc-epitope) (17), 12CA5 (IgG2b; anti-influenza hemagglutinin (HA) epitope tag) (47). Normal rabbit serum was used as a negative control.

GST Fusion Proteins—GST-Cbl-N (GST-Cbl-PTB Domain) and GST-Cbl-N/G306E (G306E) fusion proteins incorporating residues 1–357 of human Cbl were generated previously and purified as described (13).

Peptide Library Screening and Synthetic Peptides—The peptide library used here has been described previously (18). Synthetic peptides and phosphopeptides were synthesized as described (19). The degenerate peptide library (1 mg) was incubated with 200–300 μg of bead-immobilized GST-Cbl-N or its G306E mutant; the beads were washed twice with phosphate-buffered saline, and the bound phosphopeptides were eluted with 20 mM phenyl phosphate and analyzed as described previously (18). The following peptides, corresponding to ZAP-70 sequences, were used for competition experiments: pY943-NH₂-KAL-GADDSSyPTTARSAGK-COOH; pY492pY493-NH₂-KALGADDSpYPYTARSAGK-COOH; pY292NH₂-IDTLNSDGPYTPERAPICOOH; Y292-NH₂-IDTLNSDGyYTPERAPICOOH (where pY indicates phosphotyrosine); see also Table I.

cDNAs—The Lck mammalian expression vectors, pdKCR-Lck and pdKCR-Lck-Y505F (20), were kindly provided by Dr. Y. Minami (Institute for Molecular and Cellular Biology, Osaka University, Japan). CDS-Δ chimera was assembled by BamHI site-mediated ligation from two polymerase chain reaction fragments encoding human CDSΔ extra-cellular and transmembrane regions (residues 1–188) (21) and the human TCR-ζ chain cytoplasmic tail (residues 31–142 of mature polypeptide) (22) and cloned as a Xhol/Not fragment into the pSRneo vector (23). The BamHI site introduced two extra amino acids (Gly-Ser) at the CDS-Δ junction.

Generation of Myc-tagged ZAP-70 and ZAP-70 Mutants—The C-terminally myc tagged ZAP-70 construct (ZAP-70-Myc) was generated by a polymerase chain reaction from a wild type CD16:CD7:ZAP70-Myc vector (23). The Myc-tagged ZAP-70 and ZAP-70 mutants were generated by direct cloning of the PCR fragment into the pSRneo vector (see also Table I).

GST Fusion Proteins—OS-7 cells were transfected with degenerate residues at the C-terminal amino acids, usually at +1 to +3 positions (26), whereas the recently described PTB domains bind phosphotyrosine in the context of N-terminal amino acids. Although Cbl-N lacks any significant primary sequence homology to Src homology 2 or known PTB domains (27–30, 33), its Cbl-PTB domain showed strongest selection for residues immediately N-terminal to the phosphotyrosine. Notably, the Y-2 position showed a slight enrichment at Y-1, Y₂, and Y₃ positions relative to the Tyr(P) residue. The results are an average of values from two independent peptide screens. The dashed lines correspond to arbitrary conservative gates of significance at a relative enrichment of 1.5 to aid in comparison.

FIG. 1. The Cbl-PTB domain selects for phosphopeptides with a D(N/D)XpY motif. GST-Cbl-N was incubated with the degenerate phosphopeptide library, and specifically bound peptides were sequenced. The y axes (Value) depict the relative abundance of a particular amino acid compared with its expected level in the absence of any selection ("Experimental Procedures"). Y-3, Y-2, Y-1, Y+1, Y+2, and Y+3 denote the amino acid positions relative to the Tyr(P) residue. The results are an average of values from two independent peptide screens. The lanes (Y-3) correspond to arbitrary conservative gates of significance at a relative enrichment of 1.5 to aid in comparison.

REFERENCES AND DISCUSSION

The Cbl-PTB Domain Selects for Phosphopeptides with a D(N/D)XpY Motif—Of the known phosphotyrosine-binding domains, the Src homology 2 domain binds phosphotyrosine in the context of C-terminal amino acids, usually at +1 to +3 positions (26), whereas the recently described PTB domains bind phosphotyrosine in the context of N-terminal amino acids. Although Cbl-N lacks any significant primary sequence homology to Src homology 2 or known PTB domains (27–30, 33), its selective, phosphotyrosine-inhibitable binding to ZAP-70 in activated T cell lysates and EGFR in EGF-stimulated cells (13), suggested that it recognized Tyr(P) in the context of surrounding sequences. Therefore, we utilized a phosphopeptide library with degenerate residues at −3 to −1 and at +1 to +3 positions relative to Tyr(P) to determine if the Cbl-PTB domain binding motif was similar to or divergent from those of known phosphotyrosine binding domains (18, 31, 32, 34). The PTB domain-containing GST-Cbl-N was incubated with the phosphopeptide library, and the sequences of specific bound peptides were analyzed. To determine the amino acid preference, the relative abundance of various amino acids at each position was calculated (18) (Fig. 1).

The Cbl-PTB domain showed strongest selection for residues N-terminal to the phosphotyrosine. Notably, the Y-2 position was dominated by Asn (4.11-fold) followed by Asp (1.72-fold), and Asp was also strongly favored at Y-3 (2.39-fold) (Fig. 1, top). None of the amino acids showed a greater than 1.65-fold selection at the C-terminal positions relative to Tyr(P) (serine showed a slight enrichment at Y-1, Y-1, Y+1, and Y+3 positions). The loss-of-function point mutant of GST-Cbl-N (G306E)
Fig. 2. A phosphopeptide corresponding to the Tyr(P)\(^{292}\) in vivo negative regulatory site on ZAP-70 specifically inhibits ZAP-70 binding to GST-Cbl-N. Binding reactions were carried out by incubating lysates of 2.5 × 10⁷ unstimulated (−) or anti-CD3-stimulated (+) Jurkat cells with 20 μg GST-Cbl-N, GST-Cbl-N/G306E, or GST for 4 h, and binding reactions or anti-ZAP-70 immunoprecipitates (αZAP-70 LP) from 1 × 10⁶ cells were subjected to SDS-PAGE, transferred to PVDF membranes, and immunoblotted with anti-Tyr(p) antibody, followed by protein A-horseradish peroxidase and detection by enhanced chemiluminescence. For peptide competition, indicated concentrations of peptides (Table I for sequence) were added to GST fusion protein beads and lysate immediately prior to mixing. pY, Tyr(P).

...did not show any amino acid preference (data not shown). Together, these data suggested a core Cbl-PTB domain-binding motif of D(N/D)XpY. The nature of screening methodology does not allow an a priori determination of whether both D at Y-3 and N/D at Y-2 are concurrently required or if either one alone would be sufficient. As described below, a motif with Ser instead of Asp at Y-3 position is able to bind to the Cbl-PTB domain.

...The selection of N-terminal residues is reminiscent of the selectivity observed with other PTB domains (27–30). However, the preference for Ser at the Y-3 position and N/D at the Y-2 position distinguishes the Cbl-PTB domain from Shc and IRS-1 PTB domains that select an NPXpY motif. Interestingly, the Drosophila Numb PTB domain, which shows primary sequence homology to the Shc PTB domain, also showed a preference for a distinct amino acid at the Y-2 position with a consensus of GpY(X) (35). Thus, the characteristics of the identified phosphopeptide motif are consistent with a PTB domain-like mode of binding for the Cbl-PTB domain. Consistent with this idea, the D(N/D)XpY motif is predicted to have a β-turn structure in solution similar to that shown or predicted for other known PTB domain ligands (36). However, confirmation of this premise must await structural studies of the Cbl-PTB domain.

A Phosphopeptide Corresponding to the in Vivo Negative Regulatory Tyr\(^{292}\) Site on ZAP-70 Specifically Inhibits ZAP-70 Binding to the Cbl-PTB Domain—The major sites of tyrosine phosphorylation on ZAP-70 induced by TCR ligation include Tyr\(^{292}\), Tyr\(^{492}\), and Tyr\(^{493}\) (37), and all three of these sites have been demonstrated to regulate ZAP-70 signaling ability in vivo and/or in vitro (43–46), suggesting the possibility that one of these regulatory sites may create a Cbl-PTB domain-binding site. Previous phosphopeptide analysis has demonstrated that only singly phosphorylated Tyr(P)\(^{292}\) or doubly phosphorylated Tyr(P)\(^{492}\)/Tyr(P)\(^{493}\) phosphopeptides are found in vivo at detectable levels, reflecting a requirement for Tyr(P)\(^{493}\) phosphorylation before phosphorylation of Tyr\(^{492}\) (43). Therefore, phosphopeptides were synthesized corresponding to the Tyr(P)\(^{493}\), Tyr(P)\(^{492}\)/Tyr(P)\(^{493}\), and Tyr(P)\(^{292}\) sites, and tested for their competition of GST-Cbl-N binding to ZAP-70 in lysates of anti-CD3-activated Jurkat T cells (Fig. 2). The Tyr(P)\(^{493}\) (lanes 5–7), the Tyr(P)\(^{492}\)/Tyr(P)\(^{493}\) (lanes 8–10), and the nonphosphorylated Tyr\(^{292}\) peptide (lanes 14–16) had no effect on ZAP-70 binding to GST-Cbl-N at the concentrations tested. In contrast, the Tyr(P)\(^{292}\) phosphopeptide prominently competed off ZAP-70 binding to GST-Cbl-N (Fig. 2, lanes 11 and 12) with 50% inhibition (IC\(_{50}\)) observed at 4.7 μM (based on densitometric analysis, data not shown). These data confirmed the results of the phosphopeptide library screening (a D(N/D)XpY motif versus DGpY in ZAP-70 (Table I)) and strongly suggested that the Tyr(P)\(^{292}\) on ZAP-70 represents the binding site for the Cbl-PTB domain. The failure of Tyr(P)\(^{493}\) and Tyr(P)\(^{492}\)/Tyr(P)\(^{493}\) peptides to compete off binding indicates that additional residues in the motif may contribute positively or negatively to the specificity of the extended Cbl PTB domain-binding motif.

Y292F and D290A Mutations in ZAP-70 Reduce Its Binding to the CBL-PTB Domain—To determine if the motif encompassing Tyr\(^{292}\) was the major Cbl-PTB domain-binding site on ZAP-70, Y292F, and D290A mutants (mutating either the Tyr or the Asp at Y-2 in the motif) were generated by site-directed mutagenesis. COS-7 cells were transiently co-transfected with plasmids expressing wild type ZAP-70, Y292F, and D290A mutants (mutating either the Tyr or the Asp at Y-2 in the motif) and the binding was substantially decreased specifically to wild type ZAP-70, and the binding was substantially decreased in the presence of Lck-Y505F (to activate ZAP-70) (3, 6, 10). Importantly, the Tyr\(^{292}\) mutation of ZAP-70 abrogated the binding to GST-Cbl-N (Fig. 3A, top panel). This is consistent with phosphorylation and activation dependence of Cbl-N binding to ZAP-70 (13) and the requirement for cotransfected Src family kinases for full activation of ZAP-70 in COS cells (3, 6, 10). Importantly, the Y292F mutation of ZAP-70 abrogated the binding to GST-Cbl-N (Fig. 3A, top panel).

TABLE I

| Tyr position | Motif sequence |
|--------------|----------------|
| ZAP-70       | IDITLSNDDGYTPERAPIT |
| 397          | IMIQLDNFYVRLGVC |
| 492/493      | KALGADDGYTTARSAGK |
| SYK          | ESTVSFNYYEPDELAPWA |
| 523          | VMQQLNDNYVRMISIC |
| 419          | AEPLLNYKSLQRHUVK |
| 525/526      | ALRADENNYKQTHGW |
| 547          | YAPECINYYFSDKSDV |
| 630          | VEILRRNYDYDVN |
| EGFR         | PPCTIDVVMHVMKCMW |
| 998          | PSSDTNYNIALQTED |
| 1016         | DDVDAEYLIPOQGFF |
| LET-23       | PPNCSODLYQUELRCW |
| 1117         | STAQDNSLYFKTEKV |
| 1142         | LSMPDNYQNFPTSS |
| PDGFRa       | MRRVSDSNAYGVTYKE |
| 988          | PIESIFDNLYTTLSDVMS |

a Binding of Cbl-PTB domain to ZAP-70 Tyr(P)\(^{292}\) motif and its inability to bind to Tyr(P)\(^{492}\)/Tyr(P)\(^{493}\) or Tyr(P)\(^{492}\) was demonstrated here.

b SYK Tyr\(^{323}\) site and SYK Tyr\(^{292}\)/Tyr\(^{306}\) site align with ZAP-70 Tyr292 site and ZAP-70 Tyr\(^{492}\)/Tyr\(^{493}\) site, respectively.

c SYK Tyr\(^{292}\) site is near the C-terminus and ends at Y-5.

d DpY site conserved between EGFR and LET-23.

3 Z. Songyang, unpublished data.
Cbl-PTB Domain Binds ZAP-70 Negative Regulatory Site

in vivo in the context of full-length Cbl. Therefore, full-length wild type HA-Cbl or its G306E mutant were co-expressed with wild type ZAP-70 or ZAP-70/Y292F in COS cells. Lysates were prepared as in Fig. 3B, followed by anti-HA immunoprecipitation and anti-Tyr(P) immunoblotting. As seen in Fig. 3C, wild type but not the G306E mutant of Cbl associated with wild type ZAP-70 (compare lanes 2 and 3, upper panel). Notably, association of ZAP-70/Y292F with Cbl was essentially undetectable (lane 4, upper panel). Thus, both an intact Cbl-PTB domain and the Tyr(P)292 site are required for Cbl/ZAP-70 association. In the above experiments relatively similar levels of ZAP-70 or Cbl constructs were demonstrated by anti-Myc and anti-HA immunoblotting of lysates (lower two panels in Fig. 3, B and C).

Conclusion—The proto-oncogene product Cbl has recently emerged as a negative regulator of receptor and non-receptor tyrosine kinase signaling pathways (7, 11, 12, 14). The evolutionarily conserved Cbl-N region has been implicated as a crucial element in these regulatory interactions (11, 13, 14). Identification of a PTB domain within this region provided a biochemical basis for the attachment of Cbl to activated tyrosine kinases to exert a negative regulatory effect. As such, identification of a binding motif for the Cbl-PTB domain represents a significant step forward in understanding the mechanisms whereby Cbl regulates tyrosine kinases. Here, we identify a novel binding motif, D(N/D)XpY, for the Cbl-PTB domain. Our analyses directly demonstrate that the Cbl-PTB domain binds to a negative regulatory site on ZAP-70 (Tyr(P)292), which corresponds to a motif predicted by the phosphopeptide library screening.

Analysis of the sequences of selected tyrosine kinases (ZAP-70, Syk, EGFR, LET-23, and PDGFRα) known to directly interact with CBL-N and/or to be regulated by Cbl reveals the presence of potential Cbl-PTB-domain-binding motifs (Table I) (11, 24, 38, 39). Importantly, in each case one or more of the potential binding sites are known to be autophosphorylated in vivo or in vitro (3, 10, 40–42). Based on our study, we suggest that one or more of these sites may also be important for the Cbl-mediated regulation of these tyrosine kinases.

Given that the Tyr(P)292 site on ZAP-70 has been demonstrated to be an important in vivo negative regulatory site and that a candidate regulatory protein that binds this site has not been previously identified (45, 46), Cbl-PTB domain binding to this site identifies Cbl as a potential negative regulator of ZAP-70 tyrosine kinase. Consistent with this suggestion, Cbl overexpression resulted in down-regulation of Syk function (7), and we have found the Cbl-PTB domain to directly interact with activated Syk.4 We hypothesize that autophosphorylation creates Cbl-PTB-domain-binding sites on tyrosine kinases, allowing Cbl to bind and either act as a negative regulator or recruit other negative regulatory proteins.

Acknowledgments—We thank Dr. Brian Druker (Oregon Health Sciences University) for 4G10 antibody, Dr. Y. Minami (Institute for Molecular and Cellular Biology, Osaka University, Japan) for Lck cDNAs, and Dr. Brian Seed (Massachusetts General Hospital, Boston, MA) for CD16/7:ZAP-70 cDNA.

REFERENCES

1. Langdon, W. Y. (1995) Aust. N. Z. J. Med. 25, 859–864
2. Lupher, M. L., Jr., Andoniou, C. E., Bonita, D. P., Miyake, S., and Band, H. (1997) Int. J. Biochem. Cell Biol., in press
3. Wange, R. L., and Samuelson, L. E. (1996) Immunity 5, 197–205
4. Fitzpatrick, J. C., Schindler, D. G., Waks, T., and Eshhar, Z. (1997) J. Biol. Chem. 272, 8551–8557
5. Fournel, M., Davidson, D., Weil, R., and Veillette, A. (1996) J. Exp. Med. 183, 301–306
6. Latour, S., Chow, L. M. L., and Veillette, A. (1996) J. Biol. Chem. 271, 27272–27276
7. Ota, Y., and Samuelson, L. E. (1997) Science 276, 418–420

4 M. Lupher and H. Band, unpublished data.
