Tyrosine Phosphorylation of c-Cbl:

Regulation and Function

by

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ABSTRACT

Protein tyrosine kinases play an essential role within cells, transmitting signals to propagate a variety of fundamental cell functions. The activity of tyrosine kinases must be tightly regulated, however, as changes can lead to cancer, immune malfunction, and other diseases. The proto-oncogenic protein c-Cbl is a frequent substrate of a diverse array of tyrosine kinases, including receptor, cytoplasmic, and oncogenic tyrosine kinases. Interestingly, c-Cbl has multiple domains that negatively regulate tyrosine kinases. Yet. tyrosine phosphorylation of the carboxy-terminus of c-Cbl can have an activating role in signaling.

Although c-Cbl has 22 tyrosines scattered dispersed throughout it length, the carboxy-terminus of c-Cbl receives the bulk of the tyrosine phosphorylation. There, three tyrosines provide binding sites for the Src-homology 2 (SH2) domains of critical signaling intermediates. Where there is data available, reports have indicated that tyrosine kinases may be able to discriminate between these three tyrosines of c-Cbl. Given that substrate specificity of tyrosine kinases could have important ramifications for human disease, we wondered how tyrosine kinases select tyrosines within c-Cbl. We hypothesized that specificity comes from the catalytic (SH1) domains. Using purified proteins, we found that the SH1 domains of Syk, Fyn, and Abl have distinct preferences for tyrosines of c-Cbl. The specificity of each SH1 domain determined the sites of interaction between c-Cbl and SH2 domains. These data implicate SH1 domains as important

determinants of tyrosine kinase specificity and suggest that individual tyrosine kinases activate very specific pathways through c-Cbl.

One tyrosine kinase pathway that may be activated through c-Cbl is the release of inflammatory mediators from mast cells in response to antigen-bound IgE antibody. In mast cells, aggregation of the IgE receptor, FcεR1, results in activation of multiple tyrosine kinases, including Syk and Fyn. C-Cbl is temporally and spatially positioned to mediate the activation of downstream intermediates through tyrosine phosphorylation of its carboxy-terminus. We hypothesized that tyrosine phosphorylation of c-Cbl has a role in mast cell degranulation downstream of FcεR1 activation. By ectopically expressing a carboxy-terminal fragment of c-Cbl, we attempted to interfere with endogenous c-Cbl signaling and disrupt mast cell degranulation. The interfering mutant was able to inhibit degranulation but did so independently of carboxy-terminal tyrosines. Thus, we were not able to discern a role for c-Cbl tyrosine phosphorylation in mast cell function. Our work does provide, though, preliminary evidence that c-Cbl may have an activating role in FcεR1 signaling.

CHAPTER ONE

INTRODUCTION

Structure and Function of the Tyrosine Kinase Substrate c-Cbl

Allie Grossmann

c-Cbl Structure

The proto-oncogene c-Cbl was discovered by analysis of retrovirus-induced hematopoietic tumors in mice [1, 2]. An isolate of the Cas-Br-M retrovirus was found to induce B cell lymphomas and transform rodent fibroblasts. The causative oncogene, v-Cbl, was named after Casitus B-lineage lymphoma, and is a truncated form of the wild-type protein, c-Cbl.

The human c-Cbl gene codes for a 906-amino acid, 120-kDa, cytoplasmic protein. Although c-Cbl is expressed ubiquitously, the highest levels occur in hematopoietic cells and the testis [3]. The amino terminus of c-Cbl contains a unique tyrosine kinase binding (TKB) domain consisting of a four-helix bundle, a calcium-binding EF hand, and an atypical Src-homology 2 (SH2) domain (Fig. 1.1). The TKB domain, which binds phosphotyrosines of protein tyrosine kinases (PTKs), is structurally and functionally connected to a ring-finger/E3 ubiquitin ligase domain by intervening linker sequences. Together, these three regions allow c-Cbl to target PTKs for ubiquitination. Interestingly, the TKB, ring-finger and linker domains are the most highly conserved domains among Cbl family members, present in the two mammalian c-Cbl homologues, Cbl-b and Cbl-3, and the two invertebrate homologues, D-Cbl from *Drosophila melanogaster* and SLI-1 from *C. elegans* (Fig. 1.1). Similar to c-Cbl, Cbl-b is expressed ubiquitously. Cbl-3 expression, however, occurs primarily in the gastrointestinal tract.

Sequences beyond the ring-finger domain of c-Cbl act as adaptor domains (Fig. 1.2). The proline-rich region interacts with Src-homology 3 (SH3) domains of Src-family PTKs, Grb2, and Nck. Near the carboxy (C)-terminus, Src-homology 2 (SH2) recognition sequences at tyrosines 700, 731, and 774, provide docking sites for the Crk family of adaptor proteins, the p85 subunit of PI3-K, and Vav. Interestingly, Cbl-b does not have an SH2 binding site for p85, suggesting that c-Cbl may have some capacity to activate PI3-K that Cbl-b does not. Recently it has been shown that tyrosines 700, 731, and 774 are required for c-Cbl to bind the SH3 domains of CIN85 [4-6]. The contribution of these tyrosines to the interaction with the SH3 of CIN85 is not known, however, exemplifying the complexity of Cbl structure. Finally, an ubiquitin-associated domain (UBA), with no known function, and a leucine zipper (LZ) form the C-terminus of c-Cbl. Deletion of the LZ prevents homodimerization, reduces c-Cbl tyrosine phosphorylation and its association with EGFR [6, 7].

The structural diversity of c-Cbl appears to provide functional multiplicity. As described in the following sections, c-Cbl has been reported to be a scaffolding protein, fulfilling activating roles in signal transduction. Cbl can also inhibit PTK signaling through ubiquitin ligase-dependent and independent mechanisms.

c-Cbl: Inhibitor of Tyrosine Kinases

Although it was initially defined as a proto-oncogene, capable of inducing tumors in mutant form, c-Cbl has become recognized as an inhibitor of tyrosine kinase function. Beginning in 1995, a series of genetic studies established Sli-1 and D-Cbl as negative regulators of epidermal growth factor receptor (EGFR) signaling in invertebrates [8-11]. There were clues that c-Cbl might have an inhibitory function in mammalian cells but conclusive evidence was lacking [3, 12]. In 1997, Ota & Samelson [13] strengthened the case for an inhibitory role of c-Cbl by demonstrating that overexpression of c-Cbl, but not oncogenic mutants, inhibited IgE-mediated Syk activation and mast cell degranulation. The following year c-Cbl was shown to direct ligand-induced multi-ubiquitination, internalization, and degradation of growth factor and cytokine receptors [14-17], providing a mechanism for inhibition of PTK signaling. Shortly thereafter, c-Cbl was shown to have potent E3 ubiquitin ligase activity, recruiting E2 ubiquitin-conjugating enzymes through its ring-finger domain [18-20].

As the molecular mechanism of c-Cbl-mediated inhibition was being uncovered, studies of c-Cbl-deficient mice confirmed c-Cbl's role as an inhibitor of PTK signaling [21-23]. The c-Cbl-deficient mice are viable and fertile but develop lymphadenopathy, B and T cell hyperplasia, splenomegaly, extramedullary hematopoiesis, and mammary tissue hyperplasia. At the cellular level, the mice show enhanced selection of the CD4+ T cell lineage, but no alterations in negative selection. At the molecular level, $Cbl^{-/-}$ thymocytes have increased phosphotyrosine levels of Zap-70 and LAT, sustained tyrosine tyrosine

phosphorylation of SLP-76, constitutive activity of Zap-70, and increased T cell receptor (TCR) ζ chain expression. Thymocytes expressing major histocompatibility complex class II molecules also show increased surface expression of CD3 and CD4 molecules. Together, these data indicate that the inhibitory activity of c-Cbl helps set signaling thresholds during the thymic selection process that governs T cell maturation. Interestingly, peripheral T cells from $Cbl^{-/-}$ mice show reduced phosphotyrosine levels and proliferation in response to CD3 crosslinking [22], suggesting that c-Cbl has an activating role in mature T cells.

Whereas c-Cbl acts as a negative regulator in the thymus, Cbl-b negatively regulates mature T cell function [24]. Two lines of Cbl-$b^{-/-}$ mice have been generated, both are susceptible to autoimmune disease [25, 26]. The autoimmunity appears to be a result of the ability of peripheral T cells to become activated without the normal requirement of CD28 co-stimulation. Hence, the threshold for TCR activation is lowered such that mature T cells are hyperreponsive.

The phenotypes of the c-$Cbl^{-/-}$ and Cbl-$b^{-/-}$ single knockout mice suggest that the two Cbl family members are functionally distinct. However, the double knockout mice die before birth [27], indicating that there is some redundancy. Conditional inactivation of both c-Cbl and Cbl-b in mature T cells causes premature death from severe autoimmune arteritis [24].

The evidence for c-Cbl as an E3 ubiquitin ligase has continued to accumulate. Like c-Cbl, Cbl-b has also been shown to have E3 ubiquitin ligase activity [28, 29]. The inhibitory activity of the Cbl proteins is not entirely understood, however, because Cbl-mediated regulation does not always lead to changes in protein levels or internalization of receptors. In addition, the ubiquitin ligase domain is not always sufficient to suppress PTK signaling. For example, c-Cbl can inhibit c-Src through a mechanism that requires the TKB domain but not the ring finger of c-Cbl [30]. In *C. elegans*, the ring finger is partially dispensable for inhibition of LET-23 (EGFR)-mediated vulval development [31].

New discoveries have shed some light on ubiquitin ligase-independent mechanisms of inhibition by c-Cbl. For instance, the C-terminus of c-Cbl has recently been shown to promote internalization of the EGFR and the hepatocyte growth factor receptor, c-Met, by recruiting the CIN85-endophilin complex [5, 6]. Surprisingly, the interaction with the CIN85 SH3 domains is dependent on tyrosines 700, 731, and 774 of c-Cbl. Phosphorylation of these sites on c-Cbl may alter the conformation of the protein to expose polyproline sequences for SH3 binding. Alternatively, proteins such as p85 PI3-K and CrkL that bind those c-Cbl phosphotyrosines may facilitate the interaction with CIN85 [4]. Regardless, the interaction may indicate a new role for c-Cbl tyrosine phosphorylation.

Tyrosine Phosphorylation & Adaptor Functions of c-Cbl

Prior to the discovery of c-Cbl's inhibitory role in signaling, tyrosine phosphorylation was the defining feature of c-Cbl. Tyrosine phosphorylation of c-Cbl occurs rapidly and robustly upon a wide variety of cell stimuli [12]. The types of protein tyrosine kinases (PTKs) that phosphorylate c-Cbl include receptor, non-receptor, and oncogenic forms of PTKs, comprising an extensive list that is ever growing. Although c-Cbl has 22 tyrosines, the carboxy (C)-terminus of c-Cbl appears to be the major site of tyrosine phosphorylation *in vivo* [32, 33]. Specifically, tyrosines 700, 731, and 774 are heavily phosphorylated.

Tyrosine phosphorylation of c-Cbl is constitutive in various cancer cell lines and in cells expressing oncogenic PTKs [12, 34], suggesting a role for c-Cbl tyrosine phosphorylation in cancer. A role for c-Cbl in carcinogenesis is also supported by the recent finding of a Cbl translocation in a case of de novo acute myeloid leukemia [35]. While the mechanism of leukemogenesis in unknown, the translocation product is predicted to fuse the C-terminal adaptor domains of Cbl, including tyrosines 700, 731, and 774, to the amino terminus of the MLL gene.

In addition to a potential role in cancer, several studies have indicated that tyrosine phosphorylation of c-Cbl has important cellular consequences. In pro-B cells, for example, c-Cbl is inducibly tyrosine phosphorylated by interleukin-4 (IL-4) stimulation [36]. Overexpression of wild-type c-Cbl, but not a Y731F mutant, enhances IL-4 dependent survival and proliferation through activation of PI3-K. In v-Abl transformed NIH3T3 cells, c-Cbl is heavily phosphorylated and can promote adhesion and spreading only when Y700, Y731, and Y774 are intact

[37, 38]. In macrophages, integrin crosslinking induces Src-family PTK-dependent tyrosine phosphorylation of c-Cbl, spreading on fibronectin *in vitro*, and migration *in vivo* [39]. Importantly, spreading of the macrophages on fibronectin is inhibited by c-Cbl antisense oligonucleotides or by pharmacological inhibition of PI3-K. In osteoclast-like cells, Src-deficiency prevents c-Cbl tyrosine phosphorylation and bone resorption [40]. Antisense inhibition of c-Cbl reduces bone resorption as well, indicating a positive signaling role for c-Cbl downstream of Src. Interestingly, osteoclasts from c-Cbl null mice have impaired adhesion and mobility [30].

The biological functions for c-Cbl tyrosine phosphorylation suggest that the SH2-binding motifs of c-Cbl can act independently from the inhibitory activity of the TKB, linker, and ring-finger domains to act as downstream activators of PTKs. Essentially, the variety of domains within c-Cbl allows it to fulfill positive or negative regulatory roles in signaling. Whether or not c-Cbl can accomplish both functions simultaneously is not known.

The following chapters address two topics pertinent to tyrosine phosphorylation of c-Cbl. First, given that c-Cbl has numerous tyrosines scattered throughout its length, how are the C-terminal SH2 binding sites selected for tyrosine phosphorylation? Secondly, as it is known that the C-terminus of c-Cbl is inducibly tyrosine phosphorylated in response to crosslinking of the IgE receptor in mast cells, what role does the C-terminus of c-Cbl have in IgE-mediated release of inflammatory mediators?

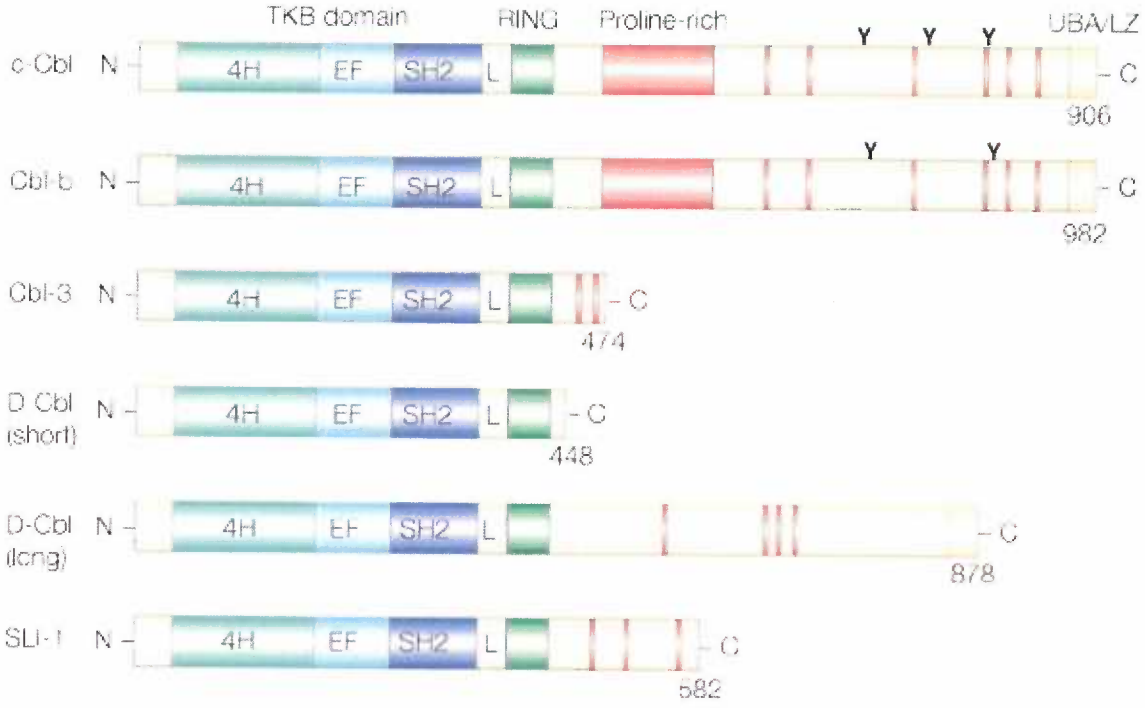

Figure 1.1: The Cbl protein family.
All homologues contain the Tyrosine Kinase Binding (TKB) domain and ring-finger (RING). The TKB is comprised of three subunits, a four-helix bundle (4H), a calcium-binding EF hand (EF), and a unique Src-homology 2 (SH2) domain. Two of the mammalian homologues, c-Cbl and Cbl-b, contain a large proline-rich region, shown in orange, in addition to the shorter polyproline sequences. Cbl-3 is also a mammalian homologue. The *Drosophila melanogaster* homologues, D-Cbl long and short, and the *Caenorhabditis elegans*, SLI-1, are also shown. The ubiquitin-associated (UBA) and leucine-zipper (LZ) domains are represented in yellow. N= amino terminus, C= carboxy terminus. Illustration courtesy of Thien and Langdon [3].

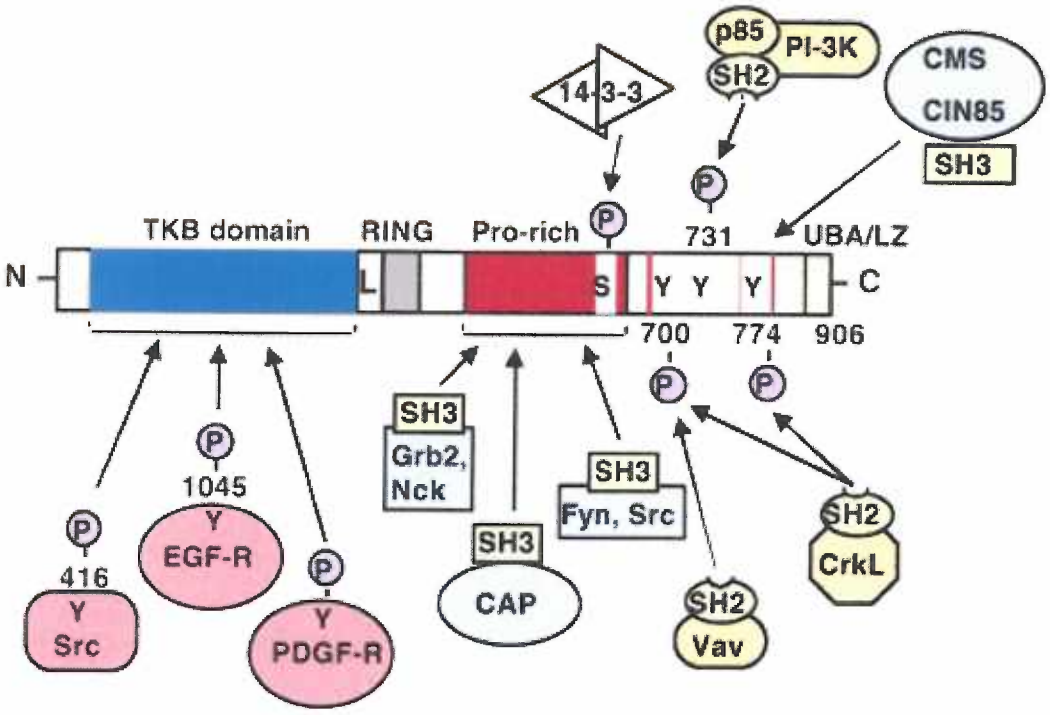

Figure 1.2: Adaptor domains and binding partners of c-Cbl.
The TKB domain interacts with phosphotyrosine binding motifs of tyrosine kinases such as Src, the epidermal growth factor receptor (EGF-R), and the platelet-derived growth factor receptor (PDGF-R). The proline-rich (Pro-rich) regions, shown in red, interact with Src-homology 3 (SH3) domains of other adaptor proteins and tyrosine kinases. Phosphotyrosines 700, 731, and 774 interact with SH2 domains. L = linker domain, RING = ring-finger, S= serine rich sequences, UBA/LZ = ubiquitin-associated/leucine zipper, P= phosphorylation. Illustration adapted from Scaife et al. [41].

CHAPTER 2

Catalytic Domains of Tyrosine Kinases Demonstrate Specificity for Tyrosines within c-Cbl

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Abstract

Substrate specificity of protein tyrosine kinases (PTKs) has largely been attributed to the ability of adaptor domains to guide the kinase to its substrate, implying that the catalytic domains of kinases have little role in selecting phosphorylation sites within their target proteins. Yet, consensus phosphorylation sites have been identified for various PTKs using peptide libraries. In addition, phosphotyrosine mapping of the PTK substrate c-Cbl, from Abl kinase-expressing cells, has indicated preferential phosphorylation of specific tyrosines. We hypothesized that the pattern of c-Cbl tyrosine phosphorylation found *in vivo* could be attributable to the specificity of the catalytic (SH1) domain of Abl, and that SH1 domains from other PTKs would also have intrinsic specificity for c-Cbl tyrosines. Our data shows that the SH1 domains of Syk, Fyn, and Abl prefer distinct tyrosines within c-Cbl and implies that phosphorylation by multiple PTKs may be required to achieve complete activation of signals mediated by c-Cbl. We propose a two-step model for PTK specificity in which SH1 domains determine the final sites of phosphorylation once the kinase reaches its target protein through adaptor domain interactions.

Introduction

PTKs are fundamental components of the communication networks within cells, transmitting signals for growth, differentiation, adhesion, motility, and death. Alteration of PTK activity can lead to a variety of human diseases, including diabetes, cancer, and immune malfunction. The proto-oncogene c-Cbl is a common substrate of PTKs and acts as both a negative regulator of and a positive effector for PTKs [3, 12].

Tyrosine phosphorylation of c-Cbl occurs rapidly and robustly in a wide variety of cell types and stimulating conditions [12]. C-Cbl is inducibly phosphorylated by receptor and nonreceptor PTKs, but constitutively phosphorylated by oncogenic PTKs and in various cancer cell lines [12, 34]. The carboxy (c)-terminus of c-Cbl contains multiple tyrosines that receive the bulk of phosphorylation relative to the rest of the protein [32, 33, 42]. Phosphorylation of three of those tyrosines, Y700, Y731, and Y774, allows the SH2 domains of Crk proteins, Vav, and the p85 subunit of phosphatidylinositol 3'-kinase (PI3-K) to bind c-Cbl.

The fact that the C-terminus of c-Cbl is the major site of phosphorylation *in vivo* suggests that either there are 3-dimensional constraints that minimize phosphorylation upstream or that PTKs have an intrinsic ability to discriminate between the 22 tyrosines of c-Cbl. Within the C-terminus, Y700, Y731, and Y774 are heavily phosphorylated [32, 33]. It has been posited that nonreceptor PTKs phosphorylate these sites indiscriminately [12]. Indiscriminate phosphorylation

suggests a lack of selectivity for individual tyrosine motifs. In contrast, studies using peptide substrates have revealed that catalytic (SH1) domains of PTKs are capable of specificity [43]. The specificity of SH1 domains may, in fact, regulate signaling outcomes involved in human disease. In multiple endocrine neoplasia type 2B, an SH1 domain mutation in the substrate-binding pocket of the RET receptor PTK was found to alter the peptide substrate preference of the kinase [44]. Clearly, more investigation into substrate specificity of SH1 domains is warranted.

Given that studies of SH1 domain specificity have been carried out exclusively with peptide substrates, the next step in the analysis is to examine specificity using a physiological substrate. c-Cbl is an ideal substrate because it is a common target for PTKs, it is a regulator of PTK signaling, and some *in vivo* sites of phosphorylation have been mapped. We asked whether SH1 domains of PTKs are able to discriminate between individual tyrosines within c-Cbl. Using purified proteins, we show that the SH1 domains of Syk, Fyn and Abl have different preferences for tyrosines within c-Cbl. Our data suggests that SH1 domains provide an additional level of specificity in PTK signaling.

Materials and Methods

In Vitro Kinase Assays - The GST-Cbl proteins were expressed in *E. coli* and purified over glutathione sepharose beads (Pharmacia). The fusion proteins were washed once in 0.5M LiCl, twice in NP40 Lysis Buffer (1% NP40, 20mM Tris pH8.0, 150mM NaCl, 10% Glycerol, 1mM EDTA, 1mM PMSF, 10mg/ml Aprotinin, 1mM Na_3VO_4), and twice in kinase buffer (see below). Bead-bound fusion proteins were resuspended in kinase buffer. All kinase buffers included 20mM Tris pH7.5, 50mM NaCl, and 1mM DTT. Syk also required 20mM $MgCl_2$ and 5mM $MnCl_2$. Fyn also required 10mM $MgCl_2$ and 10mM $MnCl_2$. Abl also required 10mM $MgCl_2$. Each kinase was used in saturating amounts. The reactions were carried out in 200mM ATP for 1 hour at room temperature. With the exception of Abl, the kinases were provided by N. Lydon (Kinetix Pharmaceuticals Inc.) as purified GST-SH1 fusions. The Abl SH1 domain was generated as previously described [45].

Gel Overlay Assays - Products of the *in vitro* kinase assays described above were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (PVDF), (Immobilon-P, Millipore). The membrane was dried at room temperature 2 hours - overnight and rehydrated by submersion in methanol and rinsing in water. The membrane was then rocked for 6-7 hours at room temperature in a 10% nonfat milk -TBST (Tris Buffered Saline + 0.05% TWEEN-20) solution, washed 3 times (10 minutes each) in TBST at room temperature, rocked overnight at 4^0C in 2mg/ml (CrkL-SH2 and p85 N+C-SH2) or 4mg/ml (p85

N-SH2) of overlay protein in binding buffer (25mM $NaPO_4$ pH 7.2, 150mM NaCl, 2.5mM EDTA pH 8.0, 20mM NaF, 1mM Na_3VO_4, 1mM DTT, 0.1% TWEEN-20, 1% nonfat milk), and washed 5 times (10 minutes each) in TBST at room temperature. Bound overlay protein was detected with either anti-Flag or anti-p85 antibodies and developed with Enhanced Chemiluminescence (Pierce) using a Roche Lumi-Imager. Quantification of signal intensity was accomplished with Roche LumiAnalyst software.

Immunoblotting - For the overlays, Flag-CrkL SH2 was detected with anti-Flag (Sigma, F3165). Both p85 N-SH2 and N+C-SH2 were detected with anti-p85 N-SH2 (Upstate Biotechnology, 05-217). PVDF membranes were stripped in 100mM β-mercaptoethanol, 2% SDS, 62.5mM Tris-HCl for 40 minutes at 65°C between immunoblots. Anti-c-Cbl (C15) and anti-GST (sc-138) were purchased from Santa Cruz Biotechnology. Anti-phosphotyrosine (4G10) was used as described [45]. Anti-mouse or rabbit IgG-HRP conjugate was purchased from Promega and used with Supersignal Chemiluminescent Substrate from Pierce to develop all immunoblots except 4G10. Anti-mouse IgG-AP conjugate was purchased from Promega and used with BCIP/NBT Color Development Substrate (Promega) to develop 4G10 immunoblots.

Plasmids - The GST-fusion constructs of Cbl were created by subcloning fragments of c-Cbl cDNA into pGEX bacterial expression vectors. NT-Cbl includes amino acids 1-220. RF-Cbl includes amino acids 144-450. PR-Cbl includes amino acids 451-541. CT-Cbl includes amino acids 542-906. The CrkL

and p85 constructs were also expressed from pGEX vectors, but were cleaved from the GST tag with Factor Xa. The CrkL-SH2 was subcloned as previously described [46] and modified by the addition of a C-terminal Flag tag. The pGEX-p85-SH2 constructs were kindly provided by T. Roberts (Dana Farber Cancer Institute).

Results

SH1 domains of PTKs exhibit distinct preferences for tyrosines within c-Cbl

In order to determine whether SH1 domains can discriminate between tyrosines of a c-Cbl, we subjected purified GST-fusion domains of c-Cbl to phosphorylation *in vitro* by the purified SH1 domains of Syk, Fyn, and Abl. Figure 1a illustrates the structure of c-Cbl and maps the regions that were expressed as GST-fusion proteins. With the exception of one tyrosine, Y141, shared between NT-Cbl and RF-Cbl, each GST-fusion domain of c-Cbl contains nonoverlapping tyrosines. In addition, every fusion protein contains one or more tyrosines, also noted in Figure 2.1a. Full-length c-Cbl was not used in our studies because it was not possible to generate the protein with sufficient purity.

As shown in Fig 2.1b-d and summarized in Table 1, all kinases tested readily phosphorylated the carboxy-terminus of c-Cbl (CT-Cbl), which includes the SH2-recognition sites Y700, Y731, and Y774. In agreement with data showing that the C-terminus of c-Cbl is the major site of tyrosine phosphorylation *in vivo* [32, 33, 42], we observed that CT-Cbl was the preferred substrate relative to other regions of c-Cbl.

The GST-CT-Cbl proteins migrate as a doublet in 10% polyacrylamide gels (uppermost bands in Fig. 2.1-2.5). GST-RF-Cbl also migrates as a doublet (Fig. 2.1). We have microsequenced the larger of the two GST-CT-Cbl bands and found it to contain both GST-CT-Cbl and bacterial Hsp70 (data not shown), suggesting that some portion of the GST-CT-Cbl migrates more slowly due to

strong interactions with the bacterial chaperone protein. Also seen in Figures 2.1-2.5 are degradation products of the GST-fusion proteins, from bacterial lysates, which copurify with the full-length proteins and migrate as a smear ahead of the full-length proteins.

The SH1 domain of Abl only phosphorylated CT-Cbl (Fig. 2.1d). In contrast, Fyn phosphorylated RF-Cbl and CT-Cbl (Fig. 2.1c) while Syk phosphorylated all of the c-Cbl regions (Fig. 2.1b). In the case of PR1-Cbl, Syk phosphorylates tyrosine 455 as it is the only tyrosine present in PR1-Cbl. Phosphorylation of tyrosine 455 was confirmed by testing the tyrosine to phenylalanine mutant version of PR1-Cbl, in which there was no phosphorylation detectable (data not shown). Phosphorylation of the GST-Cbl substrates was independent of GST, as indicated by the failure of GST to be detected by phosphotyrosine immunoblotting (Fig. 2.1b-d). The differential phosphorylation of c-Cbl supports the concept that PTKs achieve some of their substrate specificity through their SH1 domains.

Because the Y700, Y731, and Y774 residues of c-Cbl have been reported to be the primary sites of phosphorylation of c-Cbl *in vivo*, we set out to determine whether SH1 domains of PTKs could discriminate between these individual tyrosines. Again, using *in vitro* kinase assays with purified proteins, we compared the phosphotyrosine levels of wild-type (WT) GST-CT-Cbl relative to single mutants (Y700F, Y731F, Y774F) and a triple mutant (TM) in which Y700, Y731, and Y774 were mutated to phenylalanine, illustrated in Figure 2.2a.

Figures 2.2b and 2.2e show that the SH1 domain of Syk phosphorylated each of the single mutants comparably to WT while phosphorylation of the TM was substantially reduced (Fig. 2.2b, upper panel, top two bands), suggesting that Syk phosphorylates all three tyrosines with similar ability. Autophosphorylated Syk is shown in each lane except the nonphosphorylated control, which was incubated without ATP. The degradation products of the CT-Cbl proteins are phosphorylated as well and comigrate with the Syk SH1 domain (upper panel, lower bands). The lower panel, which represents total CT-Cbl protein present, was used to normalize for quantification of phosphotyrosine levels (Fig. 2.2e).

In contrast to Syk, phosphorylation by Fyn was not equal between the single mutants (Fig. 2.2c and 2.2e). In the Fyn assays, phosphorylation of Y731F was reduced relative to WT, Y700F, and Y774F. In fact, Y731F and the TM appeared to have similar levels of phosphorylation. These data suggest that Fyn prefers Y731 to other tyrosines in the C-terminus of c-Cbl. The Abl SH1 domain demonstrated yet another pattern of phosphorylation (Fig. 2.2d and 2.2e). We anticipated that Abl would phosphorylate Y700 and Y774 based on previous studies [32, 44, 47]. Mutation of Y700 significantly reduced the phosphorylation of CT-Cbl, similar to the levels of phosphorylation of the TM. Altering Y731 or Y774 had no obvious effect. The selection of Y700 by Abl is in agreement with recent data generated by mass spectrometry in which c-Cbl was

found to be phosphorylated at Y700 in Bcr-Abl-expressing cells [48]. Thus, our assay is validated by two independent studies of Abl specificity for c-Cbl *in vivo*.

Figure 2.2e summarizes the results of all *in vitro* kinase assays using the CT-Cbl proteins, all of which were repeated more than three times. We consistently observed that the SH1 domain of Syk is capable of phosphorylating Y700, Y731, and Y774 of c-Cbl. The Fyn SH1 domain prefers Y731, while the Abl SH1 domain prefers Y700. Thus, the three SH1 domains tested demonstrated distinct preferences for individual tyrosines that belong to SH2 recognition motifs of c-Cbl.

Binding of the CrkL SH2 domain to c-Cbl indicates the sites of c-Cbl phosphorylation

If SH1 domains of PTKs differentially phosphorylate c-Cbl, then binding of SH2 domains to their respective phosphotyrosine recognition motifs in c-Cbl should reflect the predicted phosphorylation pattern of the kinase. This hypothesis was tested using gel-overlay assays. Purified GST-CT-Cbl and the four tyrosine to phenylalanine mutants were phosphorylated *in vitro* by Syk, Fyn, or Abl, separated by gel electrophoresis, transferred to PVDF membranes, and incubated with the purified SH2 domain of CrkL. When Syk was used as the kinase (Fig. 2.3a), the CrkL SH2 domain readily bound to phosphorylated WT CT-Cbl. Y700 and Y774 of c-Cbl belong to known recognition motifs for the CrkL SH2 domain and, as predicted, binding of the CrkL SH2 to Y700F and Y774F CT-Cbl was reduced relative to WT and Y731F. Binding of CrkL to the TM was

completely abolished, reflecting the absence of both CrkL binding sites. The CrkL SH2 did not bind GST alone or the non-phosphorylated WT control, indicating that the interaction is dependent on phosphotyrosine. Figure 2.3a also shows equal loading of the GST-CT-Cbl proteins (middle panel) and that the phosphotyrosine levels of each protein were as expected (bottom panel). The pattern of CrkL SH2 binding to the CT-Cbl proteins was consistent in three experiments, as illustrated graphically in Figure 3d.

The binding pattern of the CrkL SH2 domain to the CT-Cbl proteins phosphorylated by Fyn was, at first, surprising. Because we had observed relatively little phosphorylation of Y700 and Y774 by Fyn, we expected minimal binding of CrkL to any of the CT-Cbl proteins. Yet, the CrkL SH2 bound to Y731F and WT (Fig. 2.3b and 2.3d). In fact, binding to Y731F was consistently higher than WT, suggesting that in our *in vitro* system, the residues targeted by Fyn shift when the preferred site, Y731, is absent. Furthermore, because the CrkL SH2 domain was able to bind WT and Y731F, there was enough phosphorylation of Y700 and/or Y774 by Fyn to mediate binding, consistent with results shown in Figure 2.2c. Nevertheless, the interaction at Y700 and Y774 is tenuous because mutation of either residue alone nearly abolishes binding of the CrkL SH2 domain to CT-Cbl. The weak nature of the interaction indicates a low level of phosphorylation and supports the conclusion that Fyn prefers Y731.

When Abl was used to phosphorylate the CT-Cbl proteins, a third pattern of CrkL SH2 binding was generated (Fig. 2.3c). Because we had observed that

Abl preferentially phosphorylates Y700, we anticipated that most of the CrkL SH2 binding would occur at Y700 of CT-Cbl. In support of this, the CrkL SH2 domain failed to bind Y700F CT-Cbl. Binding of the CrkL SH2 domain to Y774F was slightly reduced (Fig. 2.3c and 2.3d). The small reduction in binding of CrkL to Y774F suggests that there was enough phosphorylation of Y774 by Abl, albeit undetectable by phosphotyrosine immunoblotting (Fig. 2.2d and 2.3c), to mediate some of the interaction. Figure 2.3d graphically represents all three Abl experiments and supports our conclusion that the Abl SH1 preferentially targets Y700 for phosphorylation.

In summary, we observed that when the Syk SH1 domain phosphorylates CT-Cbl, the CrkL SH2 domain readily binds both Y700 and Y774, but not other sites. Fyn was able to provide docking sites for CrkL at Y700 and Y774, but the interaction was weak, reflecting Fyn's selectivity for Y731 over the CrkL SH2 binding sites. When Y731 is not present, Fyn can shift its activity towards Y700 and Y774, as suggested by the increase in binding of the CrkL SH2 to Y731F. In contrast to Fyn and Syk, phosphorylation by the Abl SH1 limits CrkL SH2 binding primarily to Y700 of CT-Cbl.

Binding of the p85 (PI3-K) SH2 domains to c-Cbl indicates the sites of c-Cbl phosphorylation

Similar to the CrkL scenario, we reasoned that if the SH1 domains of Syk, Fyn, and Abl differentially phosphorylated c-Cbl, the binding of the p85 subunit of PI3-K to CT-Cbl should also reflect the phosphorylation patterns of the kinases.

Y731 of c-Cbl belongs to a recognition motif (YxxM) for the p85 subunit of PI3-K [3]. We first tested p85N+C, containing both the amino and carboxy-terminal SH2 domains of p85, for direct interaction with CT-Cbl proteins. Figure 2.4a and 2.4d show that when the Syk SH1 domain phosphorylated the CT-Cbl proteins, p85N+C bound to WT, Y700F, and Y774F with similar affinity. Binding to the TM was abolished while some interaction occurred with Y731F, suggesting that p85N+C can bind to phosphoY700 or Y774 in addition to Y731.

The binding pattern of p85N+C to the CT-Cbl proteins phosphorylated by Fyn was identical to the binding pattern generated by Syk. Figure 2.4b shows that the strength of the interaction, with each of the CT-Cbl proteins was equivalent with the exception of Y731F, which showed a substantial reduction in binding, and the TM, which failed to bind p85N+C altogether. The reduction in binding to Y731F was expected based on our observation that the Fyn SH1 preferentially phosphorylates Y731. The complete loss of interaction with the TM is consistent with our findings from the Syk assays in which p85N+C failed to interact with the TM. Again, the fact that multiple mutations cause additional loss of interaction supports the concept that p85N+C can associate with Y700 and/or Y774, in addition to Y731.

An alternative binding-site on CT-Cbl for p85N+C was confirmed when Abl was used to phosphorylate the CT-Cbl proteins. Because Abl primarily phosphorylates Y700, binding of p85N+C to Y700F should be absent if Y700 mediates the interaction. Consistent with this assumption, Figures 2.4c and 2.4d

show that binding of p85N+C to Y700F was almost completely abrogated relative to WT. Binding to Y731F and Y774F were also reduced, although not dramatically. The binding pattern of p85N+C to the Abl-phosphorylated CT-Cbl proteins supports our conclusion that Abl preferentially phosphorylates Y700 of c-Cbl and that the SH2 domains of p85 can bind non-predicted sites in addition to Y731.

Y700 and Y774 belong to a YxxP motif and are not, therefore, predicted recognition sites for the p85 SH2 domains. Because p85 has two SH2 domains, we asked whether the ability to interact with phosphoY700 and Y774 could be ascribed to one particular SH2 domain of p85. Figure 5a and 5d show that p85-N had a reduced ability to bind Y700F and Y774F, when phosphorylated by Syk. Binding to Y731F was severely reduced. Based on these results and the observation that Syk phosphorylates all three tyrosines with similar efficiency, it appears that p85-N is capable of binding to phosphoY700 and Y774 in addition to Y731. We were unable to test the C-terminal SH2 domain of p85 because we failed to generate sufficient amounts of the purified protein.

When Fyn was used to phosphorylate the CT-Cbl proteins, a similar pattern was produced. The p85-N protein failed to bind Y731F while binding to Y700F and Y774F were reduced (Fig. 2.5b and 2.5d), providing further evidence that p85-N can bind at Y700 and Y774 in addition to Y731. Although the Fyn SH1 generates a phosphorylation pattern distinct from the Syk SH1 (Fig. 2.2b vs. 2.2c), we learned from the CrkL SH2 overlays that Fyn phosphorylates Y700 and

Y774 to some degree in our system, even though Y731 is the preferred site. Therefore, the reduction in binding of p85-N to Y700F and Y774F was not unexpected for the Fyn-mediated reactions.

Confirmation that p85-N can interact with phosphoY700 and Y774 of c-Cbl came from experiments with the Abl SH1 domain. When Abl was used to phosphorylate the CT-Cbl proteins, p85-N bound to WT, Y731F, and Y774F but failed to bind Y700F (Fig. 2.5c and 2.5d). Binding to Y774F was reduced but not as severely as Y700F. This binding pattern of p85-N indicates phosphorylation of Y700 and Y774 by the Abl SH1 domain, with Y700 being the favored site.

In summary, the SH2 domains of the p85 subunit of PI3 Kinase directly interact *in vitro* with Y731 of c-Cbl when phosphorylated by Syk and Fyn. Syk also readily phosphorylates Y700 and Y774, providing additional docking sites for p85. When Abl drives the interaction between Cbl and p85, the binding occurs mostly at Y700 but also at Y774. The ability of p85 to interact with phosphoY700 and Y774 of c-Cbl is mediated, at least in part, by the amino-terminal SH2 domain of p85.

Discussion

The mechanism by which PTKs achieve specific signaling outcomes involves adaptor domains, which guide PTKs to their substrates [49]. The role of SH1 domains in selective signaling has been considered to be minimal at best [49-51]. Yet, there is evidence that PTKs selectively phosphorylate sites within their targets. In Abl-expressing cells, for example, Y700 has been reported as a primary site of phosphorylation on ectopically expressed and endogenous c-Cbl [32, 48] while Crk appears to have only one site of phosphorylation, Y221 [51]. Both sites form the start of a YxxP motif that has been identified as the consensus phosphorylation site for Abl [44, 47]. Given that SH1 domains demonstrate preferences for peptide substrates, we reasoned that the specificity of phosphorylation observed *in vivo* resulted from the selection of particular tyrosines by the Abl SH1 domain. Broadening that concept, we hypothesized that SH1 domains, in general, confer specificity by selecting distinct tyrosines within their substrates.

We tested our hypothesis by *in vitro* kinase assay, using purified SH1 domains of PTKs and c-Cbl fragments, and then verified our findings by examining the pattern of phosphotyrosine mediated interactions between c-Cbl and downstream signaling intermediates that was generated by each PTK. We observed a preference for CT-Cbl, demonstrated by each SH1, consistent with the pattern of c-Cbl tyrosine phosphorylation found *in vivo* [32, 33, 42], suggesting that SH1 domains are the final determinants of phosphorylation sites.

To further analyze the specificity of SH1 domains for c-Cbl tyrosines, we asked whether the SH1 domains of Syk, Fyn, and Abl could discriminate between Y700, Y731, and Y774 of c-Cbl. Y700 and Y774 are located within the amino acid sequences EDEYMTP and DDGYDVP, respectively. These sequences contain a YxxP motif, similar to the consensus phosphorylation motif of Abl, IYAxP, identified by peptide library screening [44, 47]. Furthermore, Y700 and Y774 were found to be major sites of c-Cbl phosphorylation in Abl-transformed cells [32]. We anticipated, therefore, that Abl might demonstrate selectivity for Y700 and Y774 in our assays. We observed that the Abl SH1 domain can phosphorylate both residues but clearly prefers Y700. Interestingly, phosphotyrosine mapping of c-Cbl from Bcr-Abl-expressing cells, by mass spectrometry, detected phosphorylation of Y700 but not Y774 [48]. In essence, the preference we observed for Y700 by the Abl SH1 is in agreement with several independent reports of Abl specificity for c-Cbl *in vivo*, validating our assay and, again, implying that SH1 domains are the ultimate determinants of phosphorylation sites within their substrates.

A consensus phosphorylation motif has not been reported for the Fyn SH1 domain. The preferred Fyn site that we identified in CT-Cbl, Y731, lies within the sequence SCTYEAM. The EAM sequence downstream of Y731 is similar to the consensus motif identified for Syk, which includes two negatively charged amino acids followed by DYE and two uncharged amino acids [52]. The sequences neighboring Y700 and Y774 (DTEYMTP and DDGYDVP, respectively) conform

more than the Y731 sequences to the predicted Syk motif, with an adjacent D or E, negatively charged residues upstream, and uncharged residues downstream. Although the sequences are similar, they are not identical to the predicted phosphorylation motif for Syk. The ability of the Syk SH1 to phosphorylate Y700, Y731, and Y774, sites that do not exactly match its predicted peptide motif, speaks to the necessity of testing SH1 domain specificity on known protein substrates.

Feshchenko et al. [33] reported that coexpression of Syk or Fyn with c-Cbl in COS cells resulted in equivalent phosphorylation of Y700, Y731, and Y774. While our Syk data is concurrent with that of Feshchenko et al., our Fyn data is not. This discrepancy may well be due to the differences in techniques used. We used purified Fyn (SH1), providing absolute certainty that Fyn directly phosphorylates Y731. In cells, ectopic expression of full-length Fyn could have activated endogenous PTKs or Fyn may have associated with other PTKs and brought them to c-Cbl. Our use of purified proteins eliminated the potential for confounding effects from additional PTKs, a problem that exists when using immunoprecipitated proteins or *in vivo* coexpression studies.

The phosphorylation patterns we observed were confirmed in gel-overlay assays. The binding pattern of the CrkL SH2 domain, which recognizes phosphoY700 and phosphoY774, was in agreement with the predicted phosphorylation pattern of the PTK used. The binding pattern of the SH2 domains of p85 also reflected the substrate preferences of the SH1 domains.

Moreover, we observed that p85 is not limited to interacting with Y731 of c-Cbl. Simultaneous binding of both SH2 domains of p85 has been shown to result in optimal PI3-K activation [53]. Furthermore, c-Cbl has been reported to enhance PI3-K activation [36, 54]. It is possible that c-Cbl engages both SH2 domains of p85 to activate PI3-K.

Our findings imply that phosphorylation of c-Cbl by multiple PTKs may be necessary to achieve complete activation of downstream signaling pathways. Phosphorylation by either Fyn or Abl alone would be incomplete and require additional PTKs to be recruited. In T lymphocytes, Fyn and Syk, upstream activators of T cell receptor signaling [55], associate with and phosphorylate c-Cbl [27, 56], which promotes the association of c-Cbl with CrkL and PI3-K [27]. Fyn may activate PI3-K by phosphorylating Y731 of c-Cbl but would have to rely on its ability to recruit Syk to c-Cbl [56] in order for the CrkL binding sites to be phosphorylated.

Similar to Fyn, Abl is known to associate with other PTKs [57]. In v-Abl transformed fibroblasts, phosphorylation of Y700, Y731, and Y774 appears to be necessary for c-Cbl to promote fibronectin deposition, adhesion and spreading [37, 38]. While Y700, and possibly Y774, could be targets of Abl, phosphorylation of Y731 is likely to be carried out by a distinct PTK. Thus, differential phosphorylation of c-Cbl may allow for the integration of diverse upstream signals.

In summary, we have shown that SH1 domains of Abl, Syk, and Fyn differentially phosphorylate the protein c-Cbl. Fyn and Abl demonstrated striking preferences for particular sites while Syk appeared to have less specificity. The site(s) of phosphorylation selected by each SH1 domain could be loosely predicted by the consensus motifs generated from phosphopeptide library analysis. More importantly, our data are in agreement with reports of c-Cbl phosphorylation patterns *in vivo*, validating our assay and suggesting that SH1 domains can be sufficient to confer specificity when phosphorylating a target protein. To model PTK substrate specificity, we propose a two-step process in which SH1 domains determine sites of phosphorylation once a PTK has been targeted to it substrate by adaptor domain interactions. Selection of specific sites within a substrate could conceivably limit the ability of a PTK to fully trigger downstream pathways, requiring additional kinases to contribute, but would ensure that a particular pathway is consistently activated.

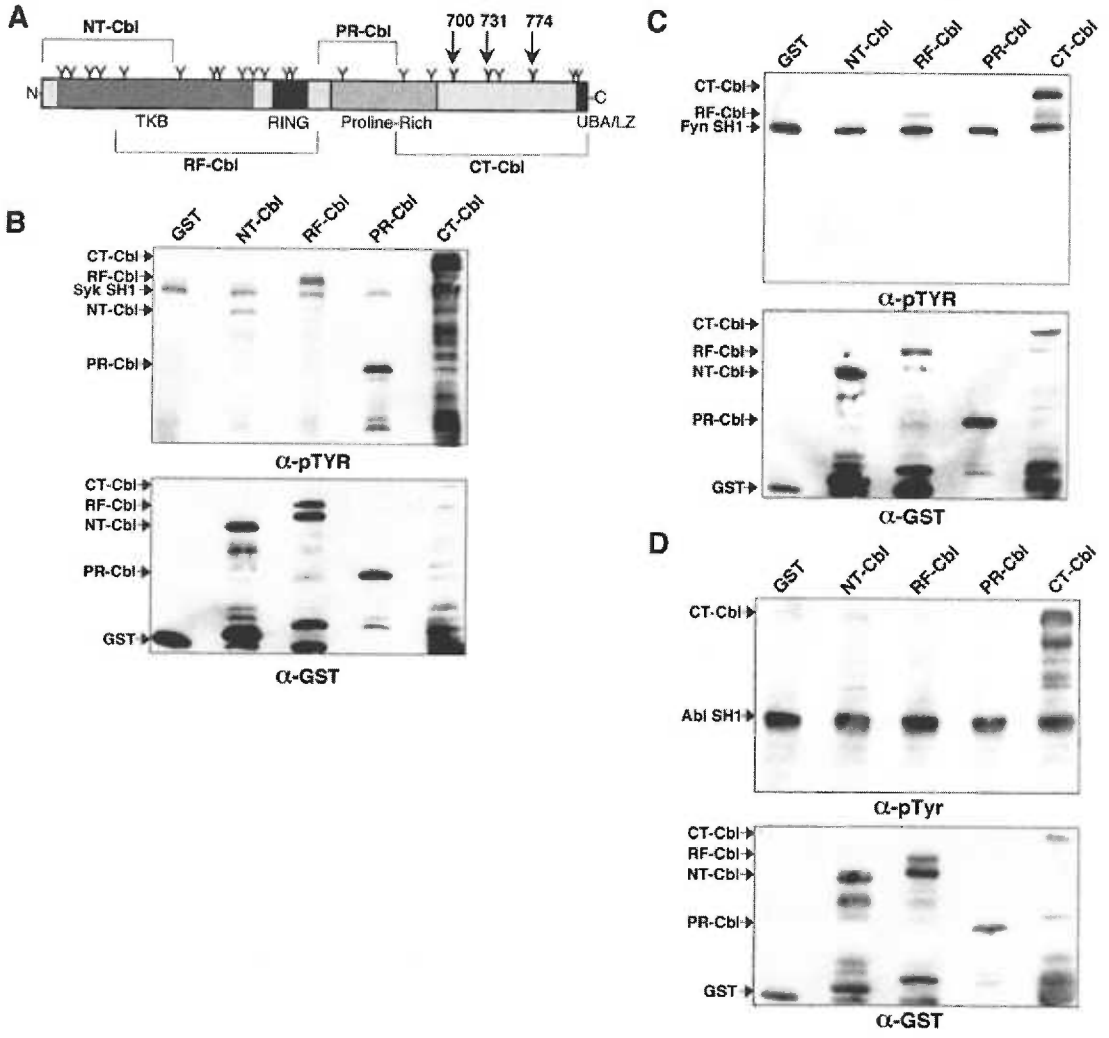

Figure 2.1: The SH1 domains of PTKs differentially phosphorylate c-Cbl domains *in vitro*. A.Structure of c-Cbl and schematic of the domains expressed as GST-fusions and used as substrates for *in vitro* kinase assays. The c-Cbl domains are designated as NT-Cbl, RF-Cbl, PR-Cbl, and CT-Cbl. Y = tyrosine, TKB = Tyrosine Kinase Binding, RING = Ring Finger, UBA = Ubiquitination site, LZ = leucine zipper, 700/731/774 = SH2 docking sites. The migration sites of the GST-Cbl fusions and the autophosphorylated SH1 domains are indicated to the left. **B-D.**Top panel = phosphotyrosine immunoblot (αpTyr), Bottom panel = GST immunoblot (αGST). Non-phosphorylated = non-P. **B.**Syk SH1 assay. **C.**Fyn SH1 assay. **D.**Abl SH1 assay.

	NT-Cbl	RF-Cbl	PR-Cbl	CT-Cbl
Syk	+	+	+	++
Fyn		+		++
Abl				++

Table 1. Comparison of c-Cbl phosphotyrosine patterns generated by the SH1 domains of Syk, Fyn, and Abl. The ability of each kinase to phosphorylate a given region of c-Cbl (labeled at the top of the columns) is designated as + where ++ = a higher degree of tyrosine phosphorylation. See Figure 1a for a description of the c-Cbl fragments.

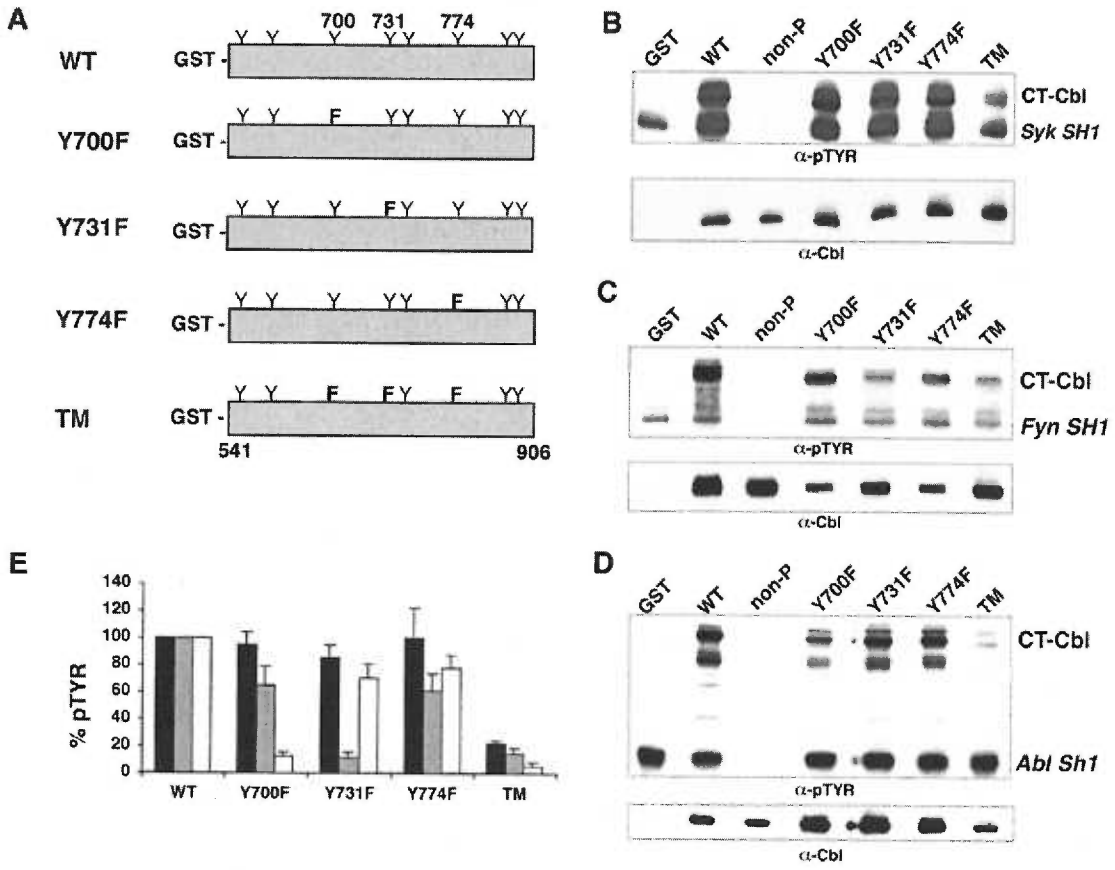

Figure 2.2: The SH1 domains of PTKs discriminate between individual tyrosines in the C-terminus of c-Cbl. A. Schematic of wild type (WT) and tyrosine (Y) to phenylalanine (F) mutants of GST-CT-Cbl which starts with amino acid 541 and ends with 906, as shown. TM = triple mutant, non-P=nonphosphorylated. For Syk (**B**), Fyn (**C**), and Abl (**D**) SH1 assays, top panel = phosphotyrosine immunoblot (α-pTyr) and bottom panel = c-Cbl immunoblot (α-Cbl). **E.**Average phosphorylation determined from 3 or more experiments, shown as % of WT. Black bars = Syk reactions, gray = Fyn, white = Abl. The phosphotyrosine levels were adjusted for loading differences. Error bars indicate standard error of the mean.

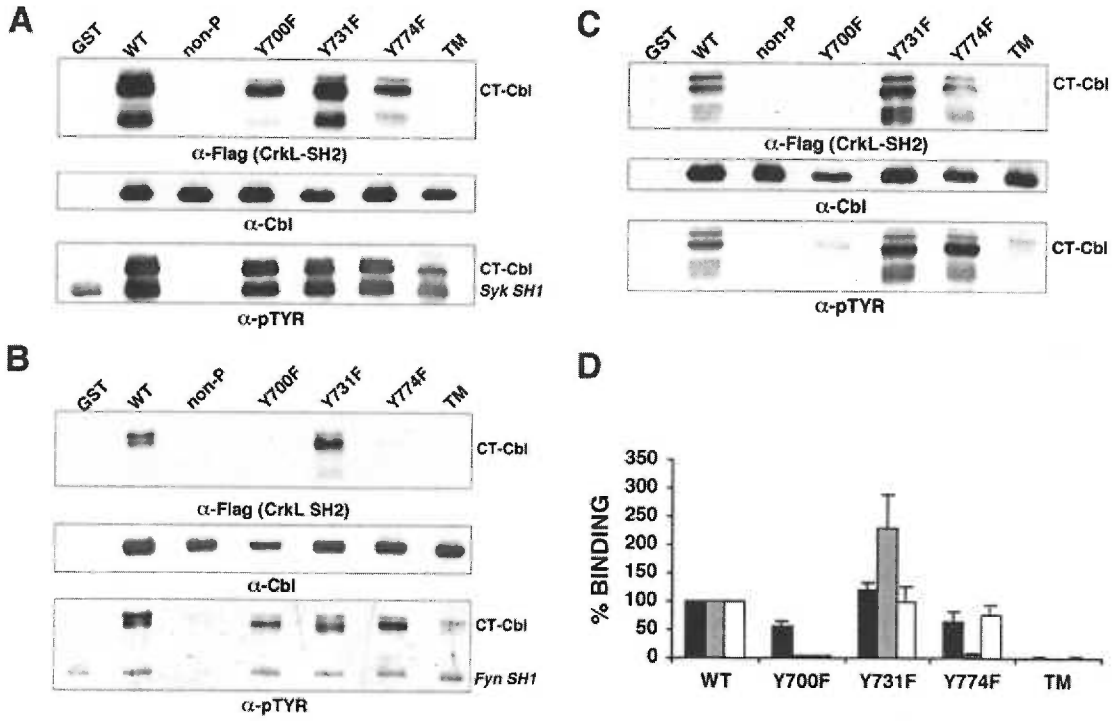

Figure 2.3: Binding of the CrkL SH2 domain to phosphorylated CT-Cbl. CrkL-SH2 gel-overlay assays. GST alone or GST-CT-Cbl proteins were phosphorylated *in vitro* by the SH1 domain of Syk (**A**), Fyn (**B**), or Abl (**C**). Top panel = overlay, detected by anti-Flag (α-Flag) immunoblot. Middle panel = anti-Cbl (α-Cbl) immunoblot. Bottom panel = anti-phosphotyrosine (α-pTyr) immunoblot. Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P = nonphosphorylated WT (no ATP in reaction). **D.**Average binding determined from 3 or more experiments, shown as % of WT. Black bars = Syk reactions, gray = Fyn, white = Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

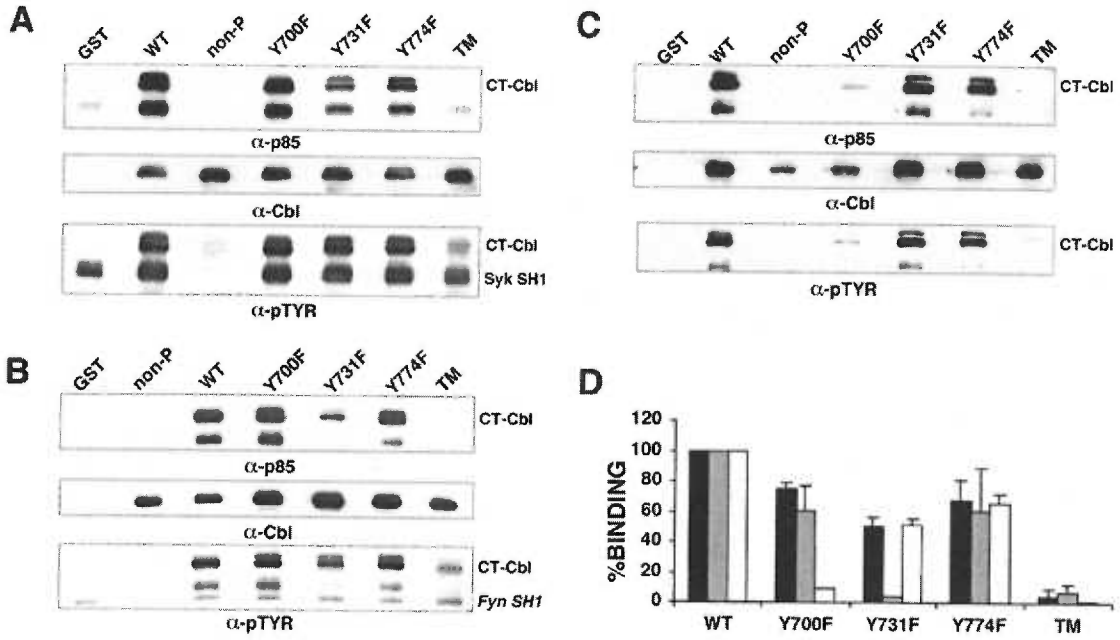

Figure 2.4 Binding of p85-N+C to phosphorylated CT-Cbl. Gel overlays of both SH2 domains of the p85 subunit of PI3-K. GST alone or GST-CT-Cbl proteins were phosphorylated *in vitro* by the SH1 domain of Syk (**A**), Fyn (**B**), or Abl (**C**). Top panel = overlay, detected by anti-p85 immunoblot (α-p85). Middle panel = anti-Cbl immunoblot (α-Cbl). Bottom panel = anti-phosphotyrosine immunoblot (α-pTyr). Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P = non-phosphorylated WT (no ATP in reaction). **D.** Average binding determined from 3 or more experiments, shown as % of WT. Black bars = Syk reactions, gray = Fyn, white = Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

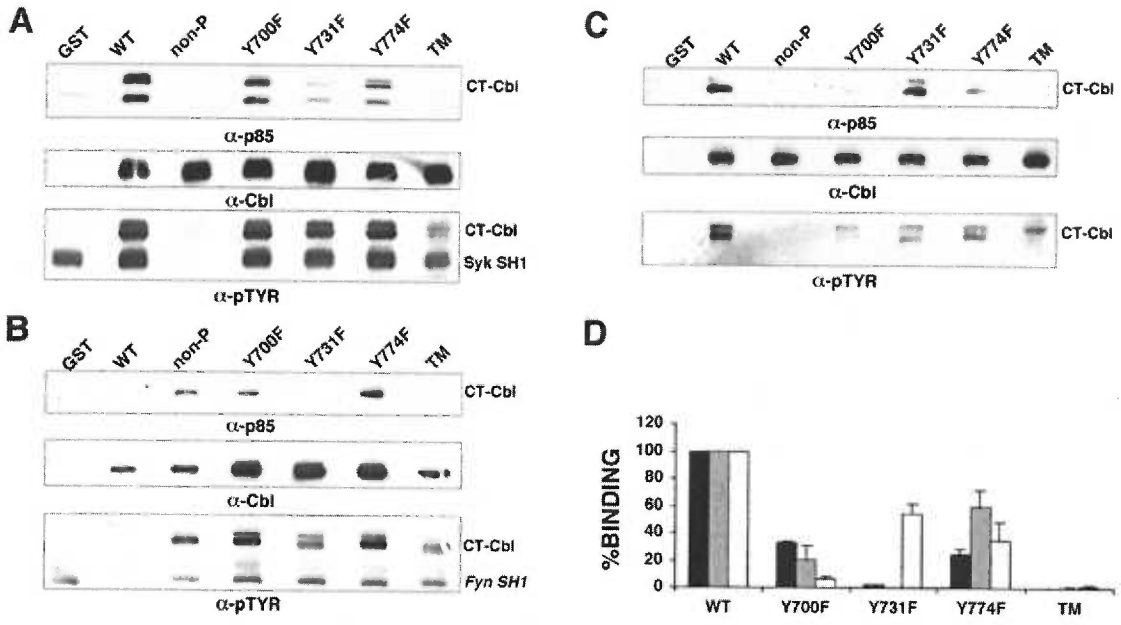

Figure 2.5:
Binding of p85-N to phosphorylated CT-Cbl. Gel-overlay assays of the amino-terminal SH2 domain of the p85 subunit of PI3-K. GST alone or GST-CT-Cbl proteins were phosphorylated *in vitro* by the SH1 domain of Syk (**A**), Fyn (**B**), or Abl (**C**). Top panel = overlay, detected by anti-p85 immunoblot (α-p85). Middle panel = anti-Cbl immunoblot (α-Cbl). Bottom panel = anti-phosphotyrosine immunoblot (α-pTyr). Substrates include GST alone (no binding detected, data not shown) and the CT-Cbl proteins listed above top panel. Non-P = non-phosphorylated WT (no ATP in reaction). **D.** Average binding determined from 3 or more experiments, with the exception of Syk (represented by 2 experiments), shown as % of WT. Black bars = Syk reactions, gray = Fyn, white = Abl. Binding intensity was adjusted for loading differences. Error bars indicate standard error of the mean.

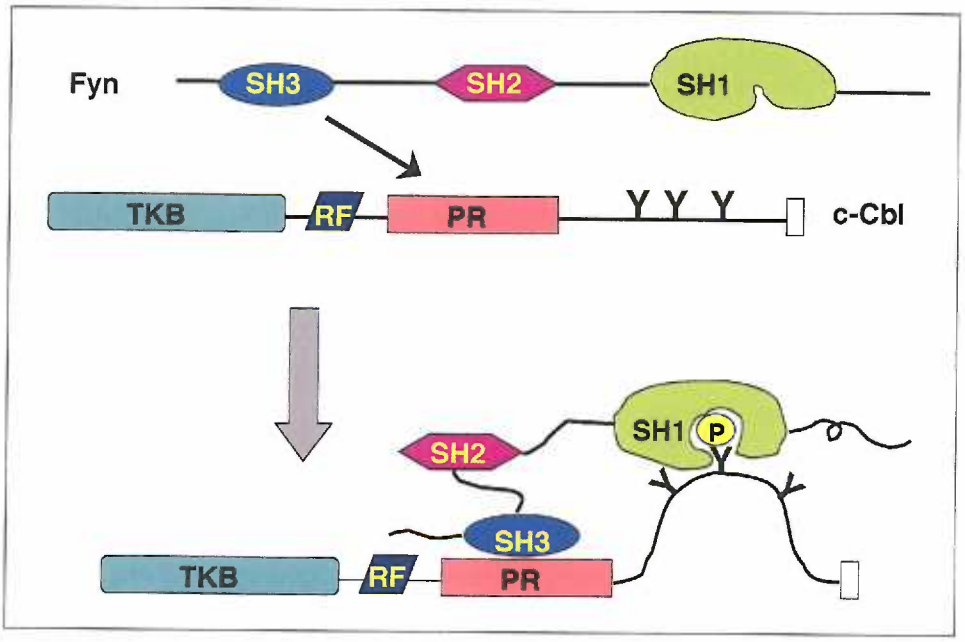

Figure 2.6: Proposed model for the mechanism of tyrosine kinase substrate specificity. Step 1; tyrosine kinases, such as Fyn, are targeted to substrates, such as c-Cbl, by adaptor domain interactions, either directly, as illustrated, or indirectly through additional associated proteins (not shown). Step 2; the catalytic domains (SH1) of tyrosine kinases discriminate among tyrosines within a substrate to determine the final pattern of phosphorylation. SH= Src homology, TKB= tyrosine kinase-binding domain, RF= ring-finger domain, PR= proline rich domain, Y= tyrosine.

Chapter 3

An Activating Role for c-Cbl in Mast Cell Degranulation

Allie H. Grossmann, Wallace Y. Langdon, Michael W. Deininger, Brian J. Druker

Abstract

Antigen-induced crosslinking of IgE-bound FcεR1 on the surface of mast cells causes the secretion of inflammatory molecules from preformed granules. Some of the earliest signaling events that occur after FcεR1 aggregation include activation of multiple protein tyrosine kinases and tyrosine phosphorylation of c-Cbl. The majority of tyrosine phosphorylation of c-Cbl is restricted to the C-terminus where phosphotyrosines provide binding sites for proteins that are known to be essential for mast cell FcεR1-regulated secretion. Localization studies have placed c-Cbl in proximity with these key signaling intermediates immediately after FcεR1 crosslinking. Hence, the C-terminus of c-Cbl may contribute to the activation of signals eminating from FcεR1 receptors that lead to secretion. We have tested this possibility by ectopically expressing a C-terminal fragment of the c-Cbl protein (CT-Cbl) to interfere with endogenous c-Cbl signaling. CT-Cbl was able to significantly inhibit secretion, but in a tyrosine-independent manner. While we were unable to show a dependence on tyrosine, we have provided preliminary data to indicate the C-terminus of c-Cbl may have an activating role in mast cell function.

Introduction

Mast cells, basophils, Langerhans cells, and monocytes express the multisubunit, high-affinity IgE receptor, FcεR1 [58, 59]. In mast cells and basophils FcεR1 governs the release of inflammatory molecules from preformed granules, a process referred to as degranulation. FcεR1 is composed of an IgE-binding α subunit and three signaling subunits: the β chain and a homodimer of γ chains. The β and γ subunits contain immune receptor tyrosine-based motifs (ITAMs), which provide docking sites for signaling intermediates. The Src family PTK Lyn is constitutively associated with the β chain. FcεR1 signaling is initiated when IgE, bound to receptors, binds multivalent antigen resulting in receptor aggregation and Lyn activation [60]. Subsequently, Lyn phosphorylates ITAM sequences on the receptor, allowing Syk to bind the γ chain and become activated [59, 61, 62]. Immediate tyrosine phosphorylation of c-Cbl ensues [42]. In addition to c-Cbl, the adaptor proteins Gab 2, Nck, Grb2, Slp-76, FYB, LAT, Shc and 3BP2 are phosphorylated, as well as the enzymes phospholipase Cγ (PLCγ), PI3-K, protein kinase C (PKC), Akt, Bruton's Tyrosine Kinase (Btk), focal adhesion kinase (FAK), the Rho family of GTPases and the Vav GTPase [63-66].

The Src family PTK Fyn has recently been reported as an additional mediator of FcεR1 signaling, associating with and phosphorylating Gab2 to regulate PI3-K activation and degranulation [67]. Mice lacking Fyn, Gab2, Vav1, LAT, SLP-76, or PLCγ2 demonstrate impaired IgE-mediated mast cell

degranulation [67-72]. Likewise, pharmacological inhibition of Syk or PI3-K antagonizes IgE-mediated secretion [73-75].

As mentioned above, c-Cbl is rapidly tyrosine phosphorylated upon FcεR1 aggregation, the majority occurring at the C-terminus [42]. It is not known, however, what role tyrosine phosphorylation of c-Cbl plays in the FcεR1 pathway. The proline-rich region of c-Cbl appears to provide a constitutive interaction with Syk in RBL2H3 mast cells [42]. As Syk does not contain an SH3 domain, the mechanism of interaction is unknown. Nevertheless, the association with Syk implicates c-Cbl as a substrate for Syk. Indeed, Syk is at least partially responsible for phosphorylating c-Cbl [42, 76]. Whether any other PTKs associate with and phosphorylate c-Cbl upon FcεR1 activation is not known.

Although a role for c-Cbl tyrosine phosphorylation in FcεR1 signaling has not been elucidated, c-Cbl appears to have an inhibitory function in mast cell degranulation. Using RBL2H3 cells, Ota and Samelson [13] demonstrated that overexpression of wild-type c-Cbl blocked Syk tyrosine phosphorylation, activation, assembly with FcεR1, and degranulation of cells. Deletion of a portion of the ring finger (70ZCbl) prevented full-length c-Cbl from inhibiting degranulation. The mechanism by which overexpression of c-Cbl inhibits secretion was clarified when it was shown that c-Cbl can mediate ubiquitination of FcεR1 and Syk [76].

The ability of c-Cbl to mediate ubiquitination of Syk is most likely facilitated by its proline-rich adaptor domain that binds Syk. Ota and Samelson [13]

showed that deletion of the proline-rich region of c-Cbl prevented full-length c-Cbl from inhibiting secretion. Deletion of the C-terminus, where most of the tyrosine phosphorylation of c-Cbl occurs, did not diminish the ability of full-length c-Cbl to inhibit secretion. Therefore, if the C-terminal tyrosines of c-Cbl have a role in FcεR1 signaling, it is likely to be distinct from the negative regulatory function carried out by upstream sequences.

A positive regulatory role for c-Cbl tyrosine phosphorylation in FcεR1 signaling is conceivable given that c-Cbl localizes, after FcεR1 activation, with Vav and the p85 subunit of PI3-K [77]. Both PI3-K and Vav have been shown to be necessary for FcεR1-mediated degranulation [71, 74, 75, 78]. In addition, p85 and Vav are known to bind to C-terminal phosphotyrosines of c-Cbl through their SH2 domains [3]. Therefore, c-Cbl may help recruit p85 and Vav to the FcεR1 signaling complex for activation.

In this report we have attempted to determine whether tyrosine phosphorylation of the C-terminus of c-Cbl has a role in IgE-mediated mast cell degranulation. Because the FcεR1 signaling pathways are identical to those in human mast cells, we used the RBL-2H3 rodent mast cell line as a model system for our studies. We found that overexpression of the C-terminus of c-Cbl was able to significantly inhibit degranulation of the RBL-2H3 cells, but in a tyrosine-independent manner. Our results indicate that the C-terminus of c-Cbl may have an activating role in FcεR1 signaling, but we could not discern a role for tyrosine phosphorylation.

Materials & Methods

Reagents

Rat basophilic leukemia (RBL) 2H3 cells were purchased from the American Tissue Culture Collection and passaged in MEM (Invitrogen) supplemented with 20% FBS (Atlanta Biologicals) and 2% glutamine. Anti-Flag antibody and multivalent dinitrophenyl conjugated to human serum albumin (DNP-HSA) was purchased from Sigma. Purified anti-2,4-dinitrophenyl (DNP) IgE was generously provided by D. Holowka (Cornell University). The pZVNeo plasmid and vaccinia virus preparations were a kind gift from G. Thomas (Vollum Institute, Oregon Health & Science University). The CT-Cbl constructs include amino acids 542-906 from human c-Cbl. W. Langdon produced WT and TM. The single mutants were generated by PCR mutagenesis. EcoR1 and Xba1 restriction sites were introduced on either end of the CT-Cbl constructs by PCR amplification for subcloning into pcDNA3 containing a Flag tag (P. Stork, OHSU). Flag-CT-Cbl cDNA was subcloned into pZVNeo at the BamH1 and Stu1 sites. The pZVNeo-CT-Cbl clones were then used to generate recombinant vaccinia virus.

Vaccinia Virus Construction and Infection

CT-Cbl vaccinia virus recombinants were generated by standard methods [79]. Prior to infection, partially purified viruses were sonicated for 10 seconds after removal from -80°C storage. Infections were carried out using 2pfu/cell in MEM supplemented with 5% FBS, 2% glutamine, and 1μg/ml anti-DNP IgE for 2 hours. Expression of each CT-Cbl virus was normalized to wild type CT-Cbl, as

determined by anti-Flag western blot, by adjusting the concentration of virus to equal wild type CT-Cbl expression.

Degranulation Assay

Degranulation was determined by β-hexosaminidase release [80]. Briefly, $2.5x10^5$ cells per well were plated in a 48-well plate, incubated overnight at 37^0C and infected with the indicated pfu/cell the following day. After infection, the cells were washed twice in Tyrode's buffer [135mM NaCl,5mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5.6mM glucose, 1mg/ml BSA, 20mM Hepes (pH 7.4)] and stimulated with 100 ng/ml DNP-HSA in Tyrode's buffer for 1 hour at 37°C. β-hexosaminidase content was quantitated as described [81]. Net degranulation (released into the media) was calculated as a fraction of the total β-hexosaminidase content within unstimulated cells.

Results

In order to test whether the C-terminus of c-Cbl has a distinct function from upstream inhibitory domains, we chose to use the isolated C-terminus of c-Cbl (CT-Cbl) for our experiments (Fig. 3.1). We reasoned that if tyrosine phosphorylation of c-Cbl has an activating role in FcεR1-mediated degranulation, expression of CT-Cbl would interfere with endogenous c-Cbl and inhibit degranulation. To this end, vaccinia virus was used to introduce Flag-tagged CT-Cbl protein into RBL2H3 cells. Initially, RBL-2H3 cells were infected with increasing amounts of either wild type (WT) or CT-Cbl (CT) virus (data not shown). A dose of 2pfu/cell was chosen for future experiments to avoid general viral toxicity to the cells. Figure 3.2a shows that CT-Cbl dramatically inhibits degranulation while the wild type virus (WT) does not. Figure 3.2b shows expression of Flag-CT-Cbl.

Once we established Flag-CT-Cbl as an interfering mutant, we asked whether its ability to inhibit degranulation is dependent on tyrosine phosphorylation of SH2 binding sites. Tyrosines 700, 731, and 774 were mutated to phenylalanine to create a triple mutant (TM) or single mutants, and then compared to wild type CT-Cbl in degranulation assays. In preliminary experiments we found that the single mutants and the TM were also able to inhibit degranulation (Fig. 3.3a), indicating that the ability of CT-Cbl to interfere with endogenous c-Cbl is independent of tyrosine phosphorylation.

Thus, ectopic expression of CT-Cbl significantly inhibits degranulation of mast cells induced by FcεR1 crosslinking. This result suggests that the C-terminus of c-Cbl has an activating role in FcεR1 signaling. The mechanism of action remains unclear, however, as mutation of tyrosines 700, 731, and 774 did not alleviate the inhibition caused by CT-Cbl.

Discussion

Our knowledge of c-Cbl function in mast cells is incomplete. Previous studies of c-Cbl in mast cells have examined localization of c-Cbl upon FcεR1 crosslinking [77] and its role as a downregulator of FcεR1 and Syk [13, 76]. The inhibitory activity of c-Cbl in FcεR1 signaling is independent of the C-terminus of c-Cbl [13], where the p85 subunit of PI3K and Vav are known to bind phosphotyrosines of c-Cbl. In RBL2H3 cells, FcεR1 aggregation induces heavy tyrosine phosphorylation of the C-terminus of c-Cbl [42] and localization of c-Cbl at crosslinked receptors with p85 and Vav [77], all within 2 minutes of cell stimulation.

Evidence suggests that more than one adaptor protein is involved in the binding and activation of PI3-K evoked by FcεR1 crosslinking. Specifically, Gab2 has been shown to be necessary, but not sufficient, for PI3-K activation and mast cell degranulation [68]. The timing of Gab2 and c-Cbl tyrosine phosphorylation after FcεR1 aggregation appears to be identical. In addition, Gab2 and c-Cbl both localize with Syk, Grb2, Vav, and p85 at patches of activated FcεR1 receptors [77]. Hence, c-Cbl is temporally and spatially positioned to activate PI3-K in parallel with Gab2.

We hypothesized that the C-terminus of c-Cbl has an activating role in FcεR1 signaling. To test our hypothesis we expressed a C-terminal interfering mutant of c-Cbl, CT-Cbl, in the RBL-2H3 mast cell line and examined the impact on degranulation. We found that CT-Cbl inhibits degranulation. Preliminary

data, however, indicates that CT-Cbl acts in a tyrosine-independent manner. While it is unclear how CT-Cbl suppresses secretion, it is not possible to make any definite conclusions about the role of c-Cbl's SH2 recognition motifs due to the artificial nature of the experimental system. Additional experiments, using c-Cbl-deficient cells, will be necessary to rule in or rule out a role for c-Cbl tyrosines (see Chapter 4, Future Directions).

Although the mechanism is unclear, our data suggests that the C-terminus of c-Cbl promotes FcεR1 signaling that governs the release of inflammatory mediators from mast cells. Our data may be the first clue that c-Cbl has an activating role in FcεR1 signaling. The broader implication for c-Cbl signaling is novel. Specifically, our findings suggest that c-Cbl can have both activating and inhibitory roles in a single pathway. In the case of FcεR1-mediated degranulation, c-Cbl may activate downstream effectors such as PI3-K, Vav, or others, while suppressing upstream Syk and FcεR1.

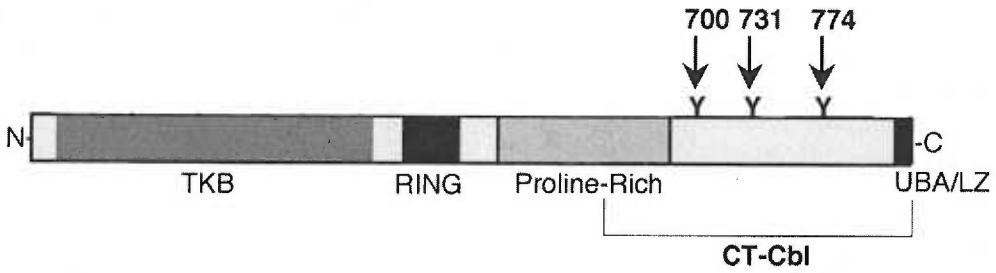

Figure 3.1: Structure of CT-Cbl relative to c-Cbl.
CT-Cbl begins in the proline-rich domain of c-Cbl at amino acid 542 and ends with the last amino acid of c-Cbl, 906. CT-Cbl contains eight tyrosines, three of which (700, 731, and 774) are noted with a Y and represent the SH2 recognition sites within c-Cbl.

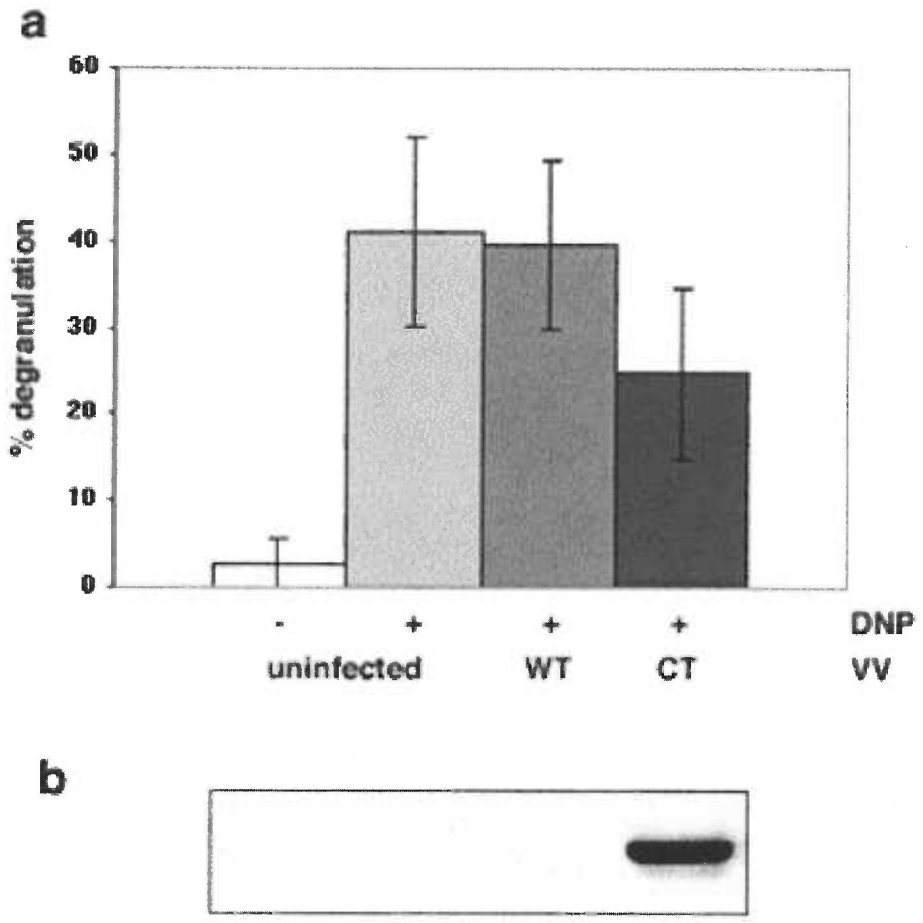

Figure 3.2: CT-Cbl inhibits degranulation.
RBL-2H3 mast cells were infected with wild-type (WT) or CT-Cbl (CT) recombinant vaccinia virus (VV) for 2 hours at 37°C or were uninfected. Cells were sensitized with anti-DNP IgE, left unstimulated (-), or stimulated (+) with 100ng/ml DNP-BSA (DNP) for 1hour. a) Degranulation is represented as the percentage of total cellular β-Hexosaminidase secreted. Error bars represent the standard deviation of 6 independent experiments. The paired *t* test was used to evaluate the statistical significance between stimulated cells that were uninfected and infected with WT virus (0.8105) or CT virus (0.0321). Values less than or equal to 0.05 indicates a statistically significant difference. b) Anti-Flag immunoblot showing expression of Flag-tagged CT-Cbl expression.

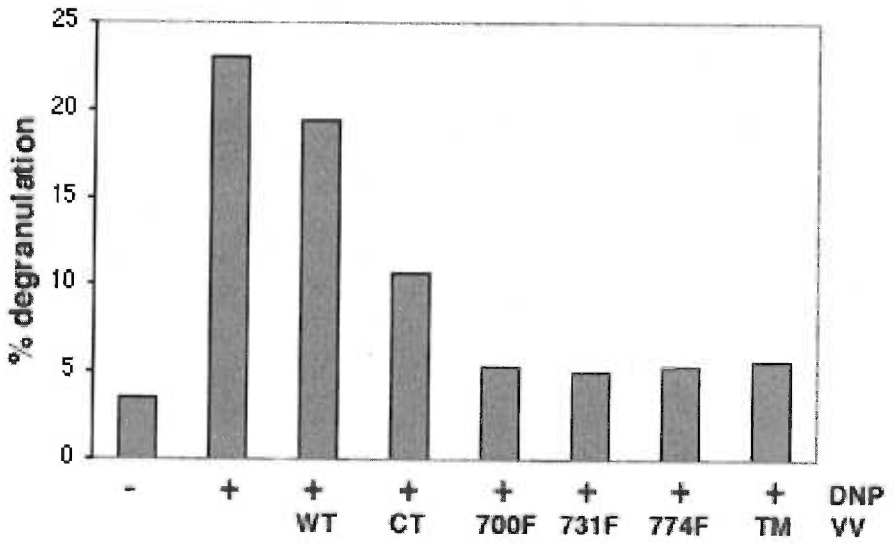

Figure 3.3: Absence of tyrosine phosphorylation sites does not prevent inhibition of degranulation by CT-Cbl. RBL-2H3 mast cells were infected with wild-type (WT) vaccinia virus (VV), recombinant virus (CT, 700F, 731F, 774F, TM), or left uninfected for 2 hours at 37°C. Cells were sensitized with anti-DNP IgE, left unstimulated (-), or stimulated (+) with 100ng/ml DNP-BSA (DNP) for 1hour. Degranulation is represented as the percentage of total cellular β-Hexosaminidase secreted. CT=CT-Cbl, F=phenylalanine substitution for tyrosine, TM=700F, 731F, 774F triple mutant.

CHAPTER 4

CONCLUSIONS & FUTURE DIRECTIONS

Allie H. Grossmann

Catalytic Domains of Tyrosine Kinases Demonstrate Specificity for Tyrosines within c-Cbl

CONCLUSIONS

Substrate specificity of PTKs has been the subject of numerous studies. While it is clear that adaptor domains contribute significantly to substrate selection, the role of SH1 domains has been questioned. Although PTKs have optimal peptide substrates, the selectivity appears to be weak relative to serine/threonine kinases [49]. Furthermore, SH1 domains are capable of phosphorylating multiple unrelated substrates. Together, these data imply that the contribution of SH1 domains to specificity in signaling is insignificant. In contrast, studies have shown that a point mutation in the substrate-binding pocket of the Ret receptor PTK, found in multiple endocrine neoplasia type 2B, switches SH1 specificity towards peptide substrates that are selected by Src and Abl, and generates new tyrosine phosphorylated proteins when expressed in cells [44, 82, 83].

If SH1 domains were not selective, it stands to reason that their substrates would not show any consistent patterns of phosphorylation. For c-Cbl, this is not the case. Not only is the C-terminus the major site of phosphorylation *in vivo*, within this region it appears that Abl targets sites that facilitate binding of CrkL. While it is possible that there are physical constraints that limit phosphorylation primarily to the C-terminus of c-Cbl, selection of particular sites within that region

suggests a level of specificity on the part of the kinase. We hypothesized that SH1 domains phosphorylate c-Cbl in a specific manner.

Our hypothesis turned out to be correct. The SH1 domains of Syk, Fyn, and Abl demonstrated distinct patterns of c-Cbl phosphorylation. Our findings imply that phosphorylation of c-Cbl by multiple PTKs may be necessary to achieve complete activation of downstream signaling pathways. In T cells, for example, Syk and Fyn play key roles in T Cell Receptor (TCR)/CD3 signaling [55]. In Fyn-deficient thymocytes c-Cbl phosphorylation is absent [27], suggesting that Fyn is the major PTK responsible for c-Cbl phosphorylation. Nevertheless, both kinases associate with c-Cbl in T cells and Fyn has been shown to recruit Syk to c-Cbl [56]. Once tyrosine phosphorylated, c-Cbl associates with CrkL and PI3-K in T cells [27]. The c-Cbl-CrkL interaction has been proposed to negatively regulate Ras signaling in T cells via association with C3G and activation of Rap1 [84]. PI3-K appears to be important in T cells for survival [24], proliferation, and IL-2 secretion [85]. Our data imply that the association of Fyn with c-Cbl results in phosphorylation of Y731 of c-Cbl and subsequent binding of PI3-K. Fyn alone would not be sufficient, however, to phosphorylate c-Cbl. Recruitment of Syk would allow for phosphorylation of Y700 and Y774, enabling CrkL to bind c-Cbl.

It is possible that SH1-mediated selectivity adds a second level of specificity to PTK signaling, determining the final phosphorylation sites once the PTK has been targeted to its substrate by adaptor domain interactions. Although

selectivity on the part of SH1 domains would limit the number of downstream mediators that are directly activated by the kinase, specificity would ensure that a particular pathway is consistently triggered.

The mechanism by which SH1 domains achieve substrate selectivity is likely to involve the active site cleft and the activation loop. The active site contains a deep pocket that binds nucleotide and peptide substrate, which binds near the γ-phosphate of ATP [86]. In the inactive state, the activation loop retains a conformation that blocks the active site from binding nucleotide and peptide substrate. Upon phosphorylation, the activation loop becomes extended, allowing access to the active site and providing a platform for the peptide substrate. The fact that the active site and activation loop both appear to interact with substrate suggests that there could be structural constraints of each that contribute to selection of specific sites within a target protein.

FUTURE DIRECTIONS

Future studies will be aimed at determining whether the patterns of c-Cbl phosphorylation produced by Syk and Fyn, in our assays, occur *in vivo*. It will be particularly important to resolve the discrepancy between our Fyn data and that of Feshchenko et al. [33], who reported a more nonspecific pattern of c-Cbl phosphorylation than we observed. Their results may reflect problems inherent in attempting to determine *in vivo* sites of phosphorylation. It may be difficult to activate Fyn without activating additional PTKs. We will attempt to avoid that possibility by using dominant negative mutants and/or RNAi, preventing endogenous Fyn activation, for example, and then examining changes in c-Cbl phosphorylation.

The mast cell line RBL-2H3 may be a useful for studying Fyn phosphorylation of c-Cbl. Fyn has recently been shown to play a critical role in FcεR1-mediated mast cell degranulation downstream of Syk [67]. Fyn associates with and phosphorylates Gab2 [67]. Gab2 colocalizes with c-Cbl to activated FcεR1 receptor complexes [77]. It is possible, therefore, that c-Cbl is also a substrate of Fyn in FcεR1 signaling.

In addition to the analysis of *in vivo* specificity, we will explore our model for PTK substrate specificity (Fig. 2.6) in more detail. The model implies that a SH1 domains act independently of the noncatalytic regions of the kinase once in contact with substrate. In order to address this, we will compare site specificities of SH1 domains with full length PTKs. We will also swap adaptor domains

between PTKs to see if noncatalytic regions can influence SH1 specificity *in vitro*. For example, adaptor domains from Syk, Fyn, and Abl will be interchanged and the effects on site-specific phosphorylation of c-Cbl will be examined. Similar experiments have been carried out *in vivo*. Mayer and Baltimore [50] reported that exchanging adaptor domains alters the protein partners that interact with the kinase within the cell, thereby changing the substrates. Conducting the domain-switching experiments with purified proteins allows us to ask a different question. With our *in vitro* system, there are no alternate substrates. Furthermore, each PTK in question is known to interact with c-Cbl so exchanging adaptor domains will not prevent the kinase from binding c-Cbl. Thus, we will be able to ascertain whether noncatalytic regions of PTKs can influence the selection of specific sites in c-Cbl by SH1 domains.

Future studies also include determining the structural components of SH1 domains that contribute to specificity. The active site cleft and the activation loop are the first places to analyze because they are thought to directly interact with substrate. It would be interesting to change the sequence of these particular regions of Syk, Fyn, or Abl to mimic one another and analyze the effects of those alterations on specificity for c-Cbl tyrosines.

Finally, we will attempt to address the biological significance of differential phosphorylation of c-Cbl. It is intriguing that the SH1 domains of Abl and Fyn had such striking preferences for Y700 and Y731 of c-Cbl, respectively. It has long been known that the consensus phosphorylation motif for Abl, YxxP,

matches the SH2 binding site of one of its major cellular targets, CrkL. CrkL is a critical mediator of Abl signaling. Likewise, Fyn may select the YxxM motif that binds the SH2 of p85 in order to ensure activation of PI3-K. It would be interesting to examine the impact of a c-Cbl Y731F mutant in the T cell receptor signaling pathway in which Fyn is a major upstream mediator. This could be accomplished by creating a Y731F knock-in mouse and examining T cell development and mature T cell function. Also, generation of Fyn-Abl hybrid knock-in mouse lines, in which the SH1 domains of each kinase has been replaced with the other, might provide new insights into the role of SH1-mediated substrate specificity in a variety of signaling pathways.

Essentially, I have described experiments that will attempt to confirm our *in vitro* findings of SH1-mediated substrate specificity with *in vivo* studies that further examine patterns of c-Cbl phosphorylation and provide biological relevance. In addition, I have outlined a course of action that will explore the mechanism of SH1-mediated substrate specificity. Because PTKs regulate a variety of essential cell functions and are increasingly becoming effective targets of anticancer therapies, these studies are expected to provide useful insight into the mechanism of their action.

CONCLUSIONS

The data presented in chapter 3 suggests new information regarding the function of c-Cbl in FcεR1 signaling. Specifically, c-Cbl may have an activating role in addition to its previously described ability to downregulate signals emanating from FcεR1 aggregates. How is it possible that c-Cbl could have such duality? The answer lies in the structure of c-Cbl. Ota and Samelson [13] showed that the 70Z mutation in c-Cbl, located within what later came to be known as the ring finger/E3 ubiquitin ligase, prevented overexpression of full-length c-Cbl from inhibiting Syk activation and mast cell degranulation. Downstream of the ring-finger and proline-rich domains, the C-terminal sequences of c-Cbl were dispensable for inhibition. It is precisely these C-terminal sequences that we isolated, expressed in mast cells, and found to interfere with degranulation.

Although we were unable to show that C-terminal tyrosines of c-Cbl have a role in degranulation, we were also unable to rule out that possibility. Overexpression of proteins can be informative but poses limitations on the interpretation of results. The overabundance of CT-Cbl may have had detrimental effects, other than competing with endogenous c-Cbl, that are independent of tyrosine phosphorylation. The presence of multiple polyproline sequences in CT-Cbl, for example, may allow CT-Cbl to sequester SH3 domain-

containing proteins that may or may not interact with endogenous c-Cbl. Hence, the lack of a clean result from our assays does not indicate that tyrosine phosphorylation of c-Cbl isn't involved in mast cell degranulation. Rather, we are only able to assert that ectopic expression of interfering mutants is not informative. We will have to use alternative means to address our hypothesis (see below, Future Directions).

We are not the first to suggest an activating role for the C-terminus of c-Cbl. As mentioned earlier, overexpression of c-Cbl, but not the Y731F mutant, has been shown enhance IL-4-dependent survival and proliferation of pro-B cells [36]. In v-Abl-transformed fibroblasts, tyrosines 700, 731 and 774 of c-Cbl appear to mediate increases in cytoskeletal rearrangements, fibronectin deposition, adhesion and spreading [37, 38]. The ability of c-Cbl tyrosines to mediate cytoskeletal rearrangements appears to occur through the activation of small GTPases [37]. Interestingly, the Rho GTPases, Cdc42 and Rac, appear to promote FcεR1-mediated secretion of mast cells [80, 81]. Proteins that bind the C-terminal phosphotyrosines of c-Cbl and localize with c-Cbl at patches of activated FcεR1 complexes, namely Vav and PI3-K, have been shown to regulate small GTPases [12, 87]. It is possible, therefore, that binding of Vav and PI3-K to phosphotyrosines of c-Cbl facilitates activation of Rho GTPases and release of secretory vesicles.

FUTURE DIRECTIONS

Many questions remain regarding the role of the C-terminus of c-Cbl in FcεR1 signaling. Most importantly, is the C-terminus *necessary* for degranulation? Also, we have not yet addressed whether c-Cbl signals through PI3-K, Vav, or other intermediates. Initially, future studies will focus on elucidating potential pathways activated by c-Cbl as the reagents necessary for those studies are already prepared. Although the tyrosine-mutated CT-Cbl constructs were not informative in degranulation assays, they may provide new information when used to analyze signaling at the molecular level. For example, c-Cbl has been shown to inducibly interact with PI3-K in hematopoietic cells stimulated through their antigen receptors [3], but FcεR1 crosslinking in mast cells, per se, has not been examined. Therefore, we will determine whether endogenous c-Cbl inducibly interacts with PI3-K and or Vav in RBL-2H3 cells. In addition, we will express the Flag-tagged CT-Cbl proteins in RBL-2H3 cells to look for additional proteins that interact with the C-terminus of c-Cbl in stimulated cells. Unidentifiable proteins in a Flag immunoprecipitation can be subjected to mass spectrometry for analysis. Subsequently, CT-Cbl will be compared to the CT-Cbl tyrosine mutants for its ability to disrupt an interaction between endogenous c-Cbl and PI3-K or Vav.

The impact of CT-Cbl and the tyrosine mutants on PI3-K and Vav activation will also be assessed. CT-Cbl may prevent lipid product formation and Akt activation by PI3-K. CT-Cbl may also reduce tyrosine phosphorylation of

Vav, which is known to activate the guanine nucleotide exchange factor activity of Vav [87]. Regardless of whether CT-Cbl affects PI3-K or Vav activation, our system provides the means to examine c-Cbl signaling at the molecular level simply by comparing the binding partners of c-Cbl, CT-Cbl, and the TM. Hence, we may be able to gain insight into the pathways activated by c-Cbl tyrosine phosphorylation by identifying the proteins that inducibly associate with c-Cbl in stimulated cells.

An investigation into the FcεR1 signaling pathways mediated by c-Cbl will require characterization of mast cell function in c-Cbl-deficient mice. Because c-Cbl has been shown to downregulate FcεR1 and Syk, determining whether the absence of c-Cbl affects activation of the pathway will require some creativity. Typically, mast cell function in mice is analyzed at the physiological level by inducing passive cutaneous or systemic anaphylaxis with IgE and antigen, and then measuring histamine release into the blood [68]. The results of this type of analysis with c-Cbl null mice may be difficult to interpret due to confounding effects of the absence of the inhibitory activity of c-Cbl. Mast cells from c-Cbl mice may be hyperactivated because FcεR1 and Syk would not be checked by the ubiquitin ligase activity of c-Cbl, overriding any defects in activation that might have been observed otherwise. There are, however, methods of getting around this potential problem by harvesting and characterizing bone marrow mast cells (BMMCs) [67-71].

BMMCs can be cultured *ex vivo*, activated through their FcεR1 receptors, and analyzed at the molecular level or for their ability to degranulate, similar to the RBL-2H3 stable mast cell line. We will use BMMCs from c-Cbl null mice [21] to determine whether tyrosine phosphorylation of c-Cbl is necessary for degranulation. Retroviral infection will be used to complement the c-Cbl null BMMCs with wild type c-Cbl or a series of mutants, including a C-terminal truncation mutant and several tyrosine point mutants. We will use an inducible expression vector, titrating the levels of protein expressed to mimick endogenous c-Cbl levels. These constructs will be compared in degranulation assays and at the molecular level to elucidate any differences in FcεR1 signaling. If these efforts prove fruitful, generating knock-in mouse lines, which would be useful for determining a physiological role of c-Cbl tyrosine phosphorylation, can extend the experiments.

I have described a course of action that will elucidate potential pathways activated by c-Cbl tyrosine phosphorylation, downstream of FcεR1, and ultimately determine whether the C-terminus of c-Cbl plays a part in the biological response of mast cells to antigen-bound IgE.

In conclusion, the primary focus of this thesis was to examine how specificity of c-Cbl tyrosine phosphorylation is achieved and to explore whether the C-terminal tyrosines of c-Cbl could act as positive regulators of a pathway in which the upstream sequences of c-Cbl are known suppress PTK signals. To this end, work presented here describes site-specific phosphorylation of c-Cbl by

the catalytic/SH1 domains of three PTKs. These studies provide insight into the mechanism of specificity achieved by PTKs and new significance to our understanding of c-Cbl tyrosine phosphorylation. Results described in this thesis also point to a role for the C-terminus of c-Cbl in promoting mast cell secretion. While the data presented here do not indicate whether tyrosine phosphorylation of c-Cbl is involved in mast cell secretion, this thesis does describe some of the preliminary work that will lead to achieving that goal.

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