Perturbed Regulation of ZAP-70 and Sustained Tyrosine Phosphorylation of LAT and SLP-76 in c-Cbl-Deficient Thymocytes

Christine B. F. Thien, David D. L. Bowtell and Wallace Y. Langdon

*J Immunol* 1999; 162:7133-7139; ;
http://www.jimmunol.org/content/162/12/7133

**References**
This article cites 48 articles, 33 of which you can access for free at:
http://www.jimmunol.org/content/162/12/7133.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Perturbed Regulation of ZAP-70 and Sustained Tyrosine Phosphorylation of LAT and SLP-76 in c-Cbl-Deficient Thymocytes

Christine B. F. Thien,* David D. L. Bowtell,† and Wallace Y. Langdon2*

Recent studies indicate that c-Cbl and its oncogenic variants can modulate the activity of protein tyrosine kinases. This finding is supported by studies showing that c-Cbl interacts directly with a negative regulatory tyrosine in ZAP-70, and that the levels of tyrosine-phosphorylated ZAP-70 and numerous other proteins are increased in TCR-stimulated thymocytes from c-Cbl-deficient mice. Here, we demonstrate that this enhanced phosphorylation of ZAP-70 and that of two substrates, LAT and SLP-76, is not due to altered protein levels but is the consequence of two separate events. First, we find increased expression of tyrosine-phosphorylated TCR chain in c-Cbl-deficient thymocytes, which results in a higher level of ζ-chain-associated ZAP-70 that is initially accessible for activation. Thus, more ZAP-70 is activated and more of its substrates (LAT and SLP-76) become tyrosine-phosphorylated after TCR stimulation. However, an additional mechanism of ZAP-70 regulation is evident at a later time post-stimulation. At this time, ZAP-70 from both normal and c-Cbl−/− thymocytes becomes hyperphosphorylated; however, only in normal thymocytes does this correlate with ZAP-70 down-regulation and a diminished ability to phosphorylate LAT and SLP-76. In contrast, c-Cbl-deficient thymocytes display altered phosphorylation kinetics, for which LAT phosphorylation is increased and SLP-76 phosphorylation is sustained. Thus, the ability to down-regulate the phosphorylation of two ZAP-70 substrates is impaired in c-Cbl−/− thymocytes. These findings provide evidence that c-Cbl is involved in the negative regulation of the phosphorylation of LAT and SLP-76 by ZAP-70. The Journal of Immunology, 1999, 162: 7133–7139.

Signals that emanate from both the TCR and coreceptors such as CD4 are responsible for determining the fate of T lymphocytes (reviewed in Refs. 1 and 2). The TCR is a complex of membrane-spanning receptors comprising the polymorphic α and β subunits, which are noncovalently associated with the TCR chains and the γ, δ, and ε chains of the CD3 complex. In thymocytes, engagement of these receptors with cross-linking Ab leads to activation of the Fyn protein tyrosine kinase and to the subsequent phosphorylation of a limited number of intracellular substrates, of which the c-Cbl protein is the most prominent (3, 4). However, this signal fails to initiate downstream signaling events, and it is only following the aggregation of the TCR with coreceptor molecules such as CD4 that phosphorylation of multiple downstream substrates and calcium mobilization occurs (3). A key event in initiating this signaling cascade is the activation of the CD4-associated kinase Lck which, in turn, phosphorylates and activates the ZAP-70 tyrosine kinase (3, 5). ZAP-70 is a ζ-chain-associated kinase that is essential for the development of normal T cells (6–8) and phosphorylates key mediators of T cell activation such as SLP-76 and LAT (9–15).

The regulation of ZAP-70 has been extensively studied in recent years and initially involves the activation of its kinase domain by Lck through the phosphorylation of tyrosine 493 (16, 17). ZAP-70 subsequently becomes hyperphosphorylated by the autokinase activity or transkinase activity of ZAP-70 molecules on adjacent ζ-chains (2). These phosphorylation sites include tyrosines 292, 492, 597, and 598; substitution with phenylalanine at any of these sites results in prominent gain-of-function phenotypes as demonstrated by lymphokine promoter activation (18–20). From these experiments, it has been postulated that these tyrosines are required to recruit regulatory proteins that suppress ZAP-70 function and inhibit further phosphorylation of substrates. Evidence from a number of studies now indicates that c-Cbl may be one of these negative regulators of ZAP-70 through its ability to bind phosphorylated tyrosine 292 in ZAP-70 via its novel Src homology 2 (SH2) domain (4, 21–25).

The original clue that c-Cbl may function as a negative regulator of tyrosine kinases came from genetic studies in Caenorhabditis elegans that identified the Cbl homologue Sli-1 (26). More recent studies in mammalian cells have also provided evidence that c-Cbl can regulate the activity of protein tyrosine kinases. Overexpression of c-Cbl inhibits Syk kinase activity and suppresses the release of serotonin from mast cells stimulated through the high-affinity receptor for IgE (27). In Jurkat T cells, c-Cbl overexpression has been found to reduce Ras-dependent AP-1 activation following Ag receptor stimulation (28), and the treatment of cells with antisense c-Cbl enhances the activation of the Janus kinase-STAT pathway (29). Compelling evidence for mammalian c-Cbl functioning as a negative regulator of tyrosine kinase activity has also come from recent studies of c-Cbl-deficient mice (4, 24). Analysis of TCR and CD4 signaling revealed that c-Cbl−/−mice lack the phosphorylation cascade characteristic of T cells, with a significant decrease in ZAP-70 and LAT phosphorylation (4). The failure of ZAP-70 to undergo sustained phosphorylation may contribute to the defective function of T cells in c-Cbl−/−mice.

Received for publication February 3, 1999. Accepted for publication April 5, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Address correspondence and reprint requests to Dr. Wallace Y. Langdon, Department of Pathology, University of Western Australia, Queen Elizabeth II Medical Center, Nedlands, Western Australia 6907, Australia, E-mail address: wlangdon@cyllene.uwa.edu.au

2 This work was supported by grants from the National Health and Medical Research Council of Australia.

3 Abbreviations used in this paper: SH2, Src homology 2; wt, wild type; SHP-1, SH2-containing protein tyrosine phosphatase-1.
thymocytes have a large enhancement in the amount of tyrosine-phosphorylated ZAP-70 and numerous unidentified proteins compared with wild-type (wt) thymocytes.

At present, the mechanism of how c-Cbl depletion can enhance the phosphorylation of ZAP-70 and other tyrosine kinase substrates is not known. However, a crucial factor in this analysis is the increased level of cell surface TCRβ, CD3ε, and CD4 in c-Cbl−/− thymocytes (4, 24). Because the activation of ZAP-70 in thymocytes is dependent upon both its association with the TCRγ chain and the amount of available CD4-associated Lck (3), it is likely that more of these components exist in c-Cbl−/− thymocytes, resulting in more activated ZAP-70 and increased phosphorylation of its substrates. In this study, we show that this is the explanation for the enhanced phosphorylation of ZAP-70 following the stimulation of c-Cbl-deficient thymocytes. However, we also observed a sustained phosphorylation of LAT and SLP-76 in c-Cbl-deficient thymocytes at a time when ZAP-70 is normally down-regulated and no longer phosphorylating these proteins to a high level. Thus, the phosphorylation kinetics of ZAP-70 substrates are markedly perturbed in c-Cbl−/− thymocytes, indicating an additional level of deregulation that is independent of effects due to differences in receptor levels.

Materials and Methods

Mice

The generation of c-Cbl-deficient mice by gene targeting of W5.9 embryonic stem cells has been described previously (4). Mouse stocks were maintained by matings of C57BL/6 × SJL/Sv intercrosses that were homozygous for either wt c-Cbl or the c-Cbl mutation.

Thymocyte stimulation by Ab cross-linking

Single-cell suspensions of thymocytes were prepared from 6- to 7-wk-old mice at 5 × 10^7 cells/ml in RPMI 1640 supplemented with 5% FCS (RPMI/5% FCS). Biotinylated hamster anti-CD3ε (500A2) and anti-CD4 (GK1.5) Abs (PharMingen, San Diego, CA) were added to the cells at 10 μg/ml and incubated on ice for 10 min before the cells were washed once in RPMI/5% FCS. Cross-linking was conducted by adding 40 μg/ml streptavidin in RPMI 1640, and the cells were stimulated by incubation on ice or at 37°C for various times before one wash in ice-cold PBS and lysis.

Immunoprecipitations and immunoblotting

Stimulated thymocytes were lysed at 3–5 × 10^7 cells/ml in ice-cold Nonidet P-40 lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, and 1% Nonidet P-40) supplemented with 10 μg/ml aprotinin, 10 mM NaF, and 1 μg/ml each of chymostatin, leupeptin, and pepstatin. After incubating for 10 min on ice, lysates were cleared by centrifugation at 4000 × g for 8 min. Cleared lysates (1-ml aliquots) were then analyzed by immunoprecipitation and immunoblotting as described previously (30). Anti-Lck Abs were purchased from Zymed (San Francisco, CA) for immunoprecipitation and from Santa Cruz Biotechnology (Santa Cruz, CA) for blotting, anti-SLP-76 Abs (monoclonal H3) were provided by Dr. A. Chan (Washington University School of Medicine, St. Louis, MO) and by Antibody Solutions (Palo Alto, CA), anti-ZAP-70 Abs were provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD) for immunoprecipitation and-by Transduction Laboratories (Lexington, KY) for blotting, anti-LAT Abs were provided by Dr. L. Samelson, anti-TCRγ Abs were obtained from Zymed for immunoprecipitation and from Dr. A. Tsygankov (Temple University School of Medicine, Philadelphia, PA) for blotting, anti-CD4 (RM4-4) Abs were provided by PharMingen, anti-c-Cbl Abs were obtained from Transduction Laboratories, and antiphosphotyrosine (4G10) Abs were provided by Dr. B. Drucker (Oregon Health Sciences University, Portland, OR). Quantitation of p16 and phosphorylated p21 TCRγ protein levels by densitometric scanning was performed using the Computing Densitometer and ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

Immune complex kinase assays

Anti-Lck or anti-ZAP-70 immunoprecipitates from unstimulated or anti-CD3 plus CD4-stimulated thymocytes were washed three times in Nonidet P-40 lysis buffer and once in kinase buffer (20 mM MOPS buffer (pH 7.0), 5 mM MgCl2, and 5 mM MnCl2). Anti-Lck immunoprecipitates were incubated with 25 μl of kinase buffer containing 12.5 μCi [γ-32P]ATP (4000 Ci/mmol, Brescatel, Adelaide, Australia) for 10 min at room temperature with occasional mixing. The kinase reaction was stopped by the addition of 1 ml of ice-cold modified RIPA buffer (20 mM MOPS (pH 7.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) and centrifuged briefly; the supernatant containing unincorporated radioisotope was discarded. Immunoprecipitates were washed an additional three times in RIPA buffer, by which time minimal radioactivity was detected in the discarded supernatant. The immunoprecipitates were resuspended in 75 μl of 1× Laemmli sample buffer, incubated at room temperature for 10 min, and subsequently boiled for 3 min; next, the supernatant was transferred to a fresh tube. ZAP-70 immune complex kinase assays were performed as described above, but in the presence of 5 μM of cold ATP and with an addition of purified erythrocyte band 3 protein (cδb3) (from Drs. R. Wange (National Institute on Aging, Baltimore, MD) and L. Samelson) as an exogenous substrate (1 μg/immunoprecipitation) followed by analysis of the supernatants. Samples were separated by electrophoresis through a 10% SDS-PAGE gel, dried at 80°C under vacuum for 30 min, and analyzed by autoradiography.

Results

The c-Cbl-deficient thymus exhibits moderate abnormalities (4, 24). There is a slight increase in cellularity at 5 wk of age that returns to normal by 6 wk, and the percentages of CD4-CD8 double-positive and CD4 and CD8 single-positive cells are normal. The most dramatic cellular abnormality is an increase in the cell surface expression of TCRγ, CD3ε, and CD4. Thus, more thymocytes have a higher density of TCR/CD3 and CD4, which could enhance signal transduction. Consistent with this, we and others reported recently that the stimulation of c-Cbl−/− thymocytes with anti-CD3 plus CD4 Abs resulted in an increase in the amount of tyrosine-phosphorylated ZAP-70 and many other proteins compared with wt thymocytes (4, 24). To determine the reasons for these effects, we compared thymocytes from c-Cbl−/− and c-Cbl−/− mice for TCRγ chain levels and for the degree of association between ZAP-70 and TCRγ.

Increased association between ZAP-70 and TCRγ in c-Cbl-deficient thymocytes

Thymocytes from 7-wk-old mice were left unstimulated or incubated with biotin-labeled anti-CD3 and anti-CD4 Abs followed by cross-linking with avidin. Next, the thymocytes were incubated on ice for an additional 15 min or placed at 37°C for 5 min before lysis and analysis by immunoprecipitation and immunoblotting. Preliminary experiments had found that these two timepoints were optimal for capturing ZAP-70 in its hypophosphorylated and hyperphosphorylated forms, respectively (data not shown and Figs. 3–5). Immunoprecipitation with anti-TCRγ Abs and immunoblotting with anti-TCRγ or antiphosphotyrosine revealed that the c-Cbl−/− thymocytes express a markedly higher level of p16 ζ-chain compared with wt thymocytes (Fig. 1A). Quantitation by densitometric scanning showed this increase to be ~3.8-fold. Interestingly, this increase is accompanied by a disproportionately larger increase (~9.5-fold) in the amount of tyrosine-phosphorylated p21 and p23 ζ-chain isoforms in c-Cbl−/− thymocytes compared with wt thymocytes (Fig. 1, A and B). This finding indicates that c-Cbl depletion not only increases the absolute level of TCRγ but also further enhances its tyrosine phosphorylation. We also observed a decrease in the amount of detectable TCRγ following stimulation of both c-Cbl−/− and c-Cbl−/− thymocytes. A likely cause of this decrease is the ubiquitination of TCRγ chains that occurs following TCR activation, which may mask epitopes recognized by TCRγ Abs (31).

Immunoblotting of the TCRγ immunoprecipitates with ZAP-70 Abs clearly demonstrated that the increased amount of tyrosine-phosphorylated ζ-chain allowed for a greater level of ZAP-70 association (Fig. 1C). Thus, c-Cbl−/− thymocytes have more TCRγ.
the amount of Lck in c-Cbl

blotting of total lysates showed that there is a moderate increase in c-Cbl

kinase assay showed that a larger pool of Lck is activated in

cytes (4, 24). Consistent with these findings, an immune complex

C

Abs (anti-TCR)

C

1

wt and c-Cbl

activation, even though total levels of ZAP-70 are equivalent be-

chain-associated ZAP-70; therefore, more ZAP-70 is available for

activation, even though total levels of ZAP-70 are equivalent be-

 tween wt and c-Cbl−/− thymocytes (Fig. 1D).

Increase in CD4-associated Lck in c-Cbl-deficient thymocytes

In thymocytes, Lck is required for the regulation of TCR chain
tyrosine phosphorylation (5), and ZAP-70 activation is dependent
upon the amount of available CD4-associated Lck (3). Therefore,
it was of interest to examine the relative amounts of Lck and CD4-
associated Lck in c-Cbl+/+ and c-Cbl−/− thymocytes. Immuno-
blotting of total lysates showed that there is a moderate increase in
the amount of Lck in c-Cbl−/− thymocytes (Fig. 2A) and an even
greater increase in the amount of CD4-associated Lck (Fig. 2B),

presumably because of higher levels of CD4 on c-Cbl−/− thymo-
cytes (4, 24). Consistent with these findings, an immune complex
kinase assay showed that a larger pool of Lck is activated in
c-Cbl−/− thymocytes following receptor cross-linking (Fig. 2C).

These findings suggest that Lck is not limiting in its availability to
activate the increased amount of TCR chain-associated ZAP-70.

Kinetics of ZAP-70 phosphorylation are equivalent between c-

Cbl+/+ and c-Cbl−/− thymocytes

A crucial observation to emerge from these studies was the finding

that the kinetics of ZAP-70 phosphorylation did not differ between
wt and c-Cbl−/− thymocytes. This was done by comparing the
progression in ZAP-70 phosphorylation at a very early point after
stimulation (i.e., cells maintained on ice for 15 min following re-
ceptor cross-linking) with cells at a later timepoint (i.e., cells incu-
bated at 37°C for 5 min) (Fig. 3). By comparing a 1X exposure
for antiphosphotyrosine immunoblots of ZAP-70 from c-Cbl−/−
thymocytes (lanes 5 and 6) with a 5X exposure from c-Cbl+/+ thymocytes (lanes 2 and 3), it can be seen that there is an equiva-
lent increase in ZAP-70 phosphorylation between 15 min on ice
and 5 min at 37°C. Thus, even though there is markedly more
phosphorylated ZAP-70 in c-Cbl−/− thymocytes, the kinetics of its
activation and subsequent hyperphosphorylation appear unaltered.
A study examining tyrosine-phosphorylated proteins following anti-CD3 stimulation revealed that, unlike ZAP-70, the phosphorylation of substrates SLP-76 and LAT showed contrasting kinetics between c-Cbl\(^{+/+}\) and c-Cbl\(^{-/-}\) thymocytes. An examination of lysates from normal thymocytes shown in Fig. 4 revealed that at an early timepoint poststimulation (i.e., 15 min on ice, when there is a low level of ZAP-70 phosphorylation), the phosphorylation of SLP-76 is high. However, at the later time of 5 min at 37°C, the increase in ZAP-70 phosphorylation corresponds with a large decrease in SLP-76 phosphorylation. A similar effect is seen with LAT; its phosphorylation pattern is also the opposite with a large decrease in SLP-76 phosphorylation. A similar effect revealed that at an early timepoint poststimulation (i.e., 15 min on ice) and markedly less active after 5 min of stimulation at 37°C, suggesting that the absence of c-Cbl is affecting an early regulatory event that is ultimately overcome by additional mechanisms involved in the negative regulation of ZAP-70 and/or the dephosphorylation of SLP-76 and LAT. Importantly, these regulatory mechanisms do not involve altered protein levels of SLP-76, LAT, or ZAP-70 (Fig. 4, D). Im- munoprecipitations of ZAP-70, SLP-76, and LAT and immunoblotting with antiphosphotyrosine confirmed these effects (Fig. 4D), which demonstrate sustained hyperphosphorylation of SLP-76 and LAT in c-Cbl-deficient thymocytes. This is in marked contrast to normal thymocytes, which exhibit a regulatory pattern that involves a rapid rise and fall in SLP-76 and LAT tyrosine phosphorylation.

In contrast, analysis of c-Cbl-deficient thymocytes showed that during the transition from stimulation on ice to stimulation at 37°C, there is no decrease in SLP-76 phosphorylation, and remarkably the phosphorylation of LAT is increased, even though ZAP-70 phosphorylation is elevated (Fig. 4A). This contrasting pattern of tyrosine phosphorylation between c-Cbl\(^{+/+}\) and c-Cbl\(^{-/-}\) thymocytes is clearly illustrated when long and short exposures are compared for ZAP-70 and SLP-76 (Fig. 4C). Immunoprecipitations of ZAP-70, SLP-76, and LAT and immunoblotting with antiphosphotyrosine confirmed these effects (Fig. 4D), which demonstrate sustained hyperphosphorylation of SLP-76 and LAT in c-Cbl-deficient thymocytes. This is in marked contrast to normal thymocytes, which exhibit a regulatory pattern that involves a rapid rise and fall in SLP-76 and LAT tyrosine phosphorylation.

The effect on ZAP-70 activity was also examined by an immune complex kinase assay using purified erythrocyte band 3 protein (cd3) as an exogenous substrate (Fig. 5). Consistent with in vivo studies, the ZAP-70 isolated from c-Cbl\(^{+/+}\) thymocytes is most active at phosphorylating cd3 at an early point after stimulation (i.e., 15 min on ice) and markedly less active after 5 min of stimulation at 37°C. This trend provides additional proof that these stimulation procedures are capturing ZAP-70 during its transition from an active to an inactive kinase. Also consistent with the in...
compared with c-Cbl

First, there is more TCR

Discussion

By examining the progression of ZAP-70 activation and down-regulation in CD3 plus CD4 cross-linked thymocytes, we have been able to demonstrate multiple effects of c-Cbl depletion on ZAP-70 and retains the ability to phosphorylate cd3b to a high level at the later timepoint (Fig. 5, 5 min 37°C). However, this effect is not sustained indefinitely. We found that between 5 and 15 min of stimulation at 37°C, there is a marked drop in the in vitro phosphorylation of cd3b by ZAP-70, although it remains significantly higher in the c-Cbl−/− thymocytes compared with the wt thymocytes (data not shown). This decreased ZAP-70 activity is consistent with the drop in LAT and SLP-76 tyrosine phosphorylation shown in Fig. 4A.

vivo studies is the observation that ZAP-70 from c-Cbl−/− thymocytes can phosphorylate more cd3b (because there is more activated ZAP-70) and retains the ability to phosphorylate cd3b to a high level at the later timepoint (Fig. 5, 5 min 37°C). However, this effect is not sustained indefinitely. We found that between 5 and 15 min of stimulation at 37°C, there is a marked drop in the in vitro phosphorylation of cd3b by ZAP-70, although it remains significantly higher in the c-Cbl−/− thymocytes compared with the wt thymocytes (data not shown). This decreased ZAP-70 activity is consistent with the drop in LAT and SLP-76 tyrosine phosphorylation shown in Fig. 4A.

A key factor in our analysis of ZAP-70 and the phosphorylation of its substrates (SLP-76 and LAT) was the procedure of incubating thymocytes on ice following receptor cross-linking, which allowed us to examine signaling events at a very early point after stimulation. This method has been used previously to study the kinetics of c-Cbl tyrosine phosphorylation and ubiquitination in CSF-1-stimulated macrophages (32). Importantly, this allowed us to capture ZAP-70 when it is highly active and able to phosphorylate SLP-76, LAT, and cd3b to a high level (Figs. 4 and 5). This activity also correlated with ZAP-70 being hypophosphorylated, which presumably represents a predominance of ZAP-70 molecules that are singly phosphorylated on the activating tyrosine 493 (16, 17). At a later timepoint poststimulation, we found that there was a very large increase in ZAP-70 phosphorylation in both c-Cbl+/+ and c-Cbl−/− thymocytes, and that the relative increase was equivalent between both populations (Fig. 3). Thus, although more ZAP-70 is available for activation in c-Cbl−/− thymocytes, its sequential pattern of increasing phosphorylation over time appears unaltered.

However, at this later time it was apparent that there was a perturbation in the function of ZAP-70 from c-Cbl−/− thymocytes that appeared unrelated to the quantitative changes described above. In c-Cbl−/− thymocytes, the increased phosphorylation of ZAP-70 coincided with a large decrease in the phosphorylation of its substrates, SLP-76 and LAT (Fig. 4). This observation is consistent with the phosphorylation of additional tyrosine residues (i.e., 292, 492, 597, and 598), which are involved in the negative regulation of ZAP-70 (18–20). In contrast, it was clear that at this later time after stimulation, the activity of ZAP-70 from c-Cbl−/− thymocytes was markedly altered with respect to its phosphorylation of SLP-76 and LAT such that SLP-76 phosphorylation remained constant and LAT phosphorylation increased (Fig. 4). Thus, the regulation of ZAP-70 is perturbed in c-Cbl−/− thymocytes.

How c-Cbl is involved in the regulation of the phosphorylation of SLP-76 and LAT by ZAP-70 is unknown, because c-Cbl itself does not possess a known catalytic activity. However, c-Cbl has been found to associate with ZAP-70 in Jurkat T cells (21), and this interaction appears to be through its divergent SH2 domain and the negative regulatory tyrosine 292 of ZAP-70 (23). Importantly, the crystal structure of this interaction has been resolved recently (25). In this study however, we were unable to detect an in vivo interaction between c-Cbl and ZAP-70 in wt thymocytes, suggesting that the interaction may be weak or transient. Interestingly, two studies that investigated the function of tyrosine 292 in ZAP-70 both predicted that this site would interact with an inhibitory molecule to negatively regulate ZAP-70 function (18, 19). It is noteworthy that a point mutation in the SH2 domain that prevents c-Cbl binding to phosphorylated tyrosine 292 of ZAP-70 correspondingly abolishes the negative regulatory activity of the C. elegans homologue Sli-1 (22, 25, 26). Thus, the properties of c-Cbl and its C. elegans homologue are consistent with the predictions regarding this inhibitory molecule, and the c-Cbl depletion investigated here may be preventing the induction of this aspect of ZAP-70 regulation. The mechanism of how an interaction of the SH2 domain of c-Cbl with tyrosine 292 affects ZAP-70 or its substrates remains to be resolved, as does a determination of the roles of the extensive SH3 and SH2 domain-binding regions and RING finger motif of c-Cbl. It is possible that recruitment of c-Cbl binding proteins into this complex is essential for the regulatory process that involves phosphorylated tyrosine 292. The negative regulation of ZAP-70 by its tyrosine phosphorylation has also been
shown to be affected by the SH2-containing protein tyrosine phosphatase-1 (SHIP-1) (33); however, we have found no evidence of a c-Cbl interaction with SHIP-1 (data not shown). SHIP-1 can directly decrease the amount of tyrosine phosphorylated ZAP-70, which in turn reduces the ability of ZAP-70 to phosphorylate substrates. Our findings indicate that the negative regulation of ZAP-70 by c-Cbl is distinct from that of SHP-1 because it does not directly affect ZAP-70 phosphorylation; however, like SHP-1, it does alter the ability of ZAP-70 to phosphorylate substrates. How these two proteins act to coordinate the activity of ZAP-70 will be an important aspect to consider in future studies.

Our study of the kinetics of tyrosine phosphorylation also revealed that the phosphorylation of c-Cbl peaks at a later time than either SLP-76 or LAT and follows more closely the hyperphosphorylation of ZAP-70 (Fig. 4A). Therefore, this timing fits closely with c-Cbl being phosphorylated when ZAP-70 is being down-regulated and is consistent with its role as a negative regulator of T cell activation. In addition, c-Cbl is phosphorylated independently of ZAP-70, SLP-76, and LAT because it is phosphorylated by the Src kinase Fyn (34) which, unlike ZAP-70, SLP-76, and LAT, can be activated by TCR/CD3 stimulation alone (4). Although the precise role of Fyn in TCR-mediated signaling in thymocytes has yet to be resolved, the predominance of c-Cbl as a substrate suggests c-Cbl phosphorylation is a major function for this kinase.

In summary, this study provides biochemical evidence that c-Cbl is a negative regulator of ZAP-70. Furthermore, these findings help to provide a mechanism to explain the enhanced positive selection seen in the thymi of c-Cbl knockout mice that express an MHC class II-restricted transgenic TCR (6 – 8). Because signaling with c-Cbl is phosphorylated when ZAP-70 is being down-regulated and is consistent with its role as a negative regulator of T cell activation. In addition, c-Cbl is phosphorylated independently of ZAP-70, SLP-76, and LAT because it is phosphorylated by the Src kinase Fyn (34) which, unlike ZAP-70, SLP-76, and LAT, can be activated by TCR/CD3 stimulation alone (4). Although the precise role of Fyn in TCR-mediated signaling in thymocytes has yet to be resolved, the predominance of c-Cbl as a substrate suggests c-Cbl phosphorylation is a major function for this kinase.

In summary, this study provides biochemical evidence that c-Cbl is a negative regulator of ZAP-70. Furthermore, these findings help to provide a mechanism to explain the enhanced positive selection seen in the thymi of c-Cbl knockout mice that express an MHC class II-restricted transgenic TCR (6 – 8). Because signaling with c-Cbl is phosphorylated when ZAP-70 is being down-regulated and is consistent with its role as a negative regulator of T cell activation. In addition, c-Cbl is phosphorylated independently of ZAP-70, SLP-76, and LAT because it is phosphorylated by the Src kinase Fyn (34) which, unlike ZAP-70, SLP-76, and LAT, can be activated by TCR/CD3 stimulation alone (4). Although the precise role of Fyn in TCR-mediated signaling in thymocytes has yet to be resolved, the predominance of c-Cbl as a substrate suggests c-Cbl phosphorylation is a major function for this kinase.

Acknowledgments

We thank Dr. Larry Samelson for ZAP-70 and LAT Abs, Dr. Andrew Chan for SLP-76 Abs, Dr. Alex Tsygankov for TCR Abs, Dr. Brian Druker for antiphosphotyrosine Abs, and Drs. Ronald Wange and Larry Samelson for purified cd3 protein. We also thank Drs. Larry Samelson, Jeroen van Leeuwen, Robin Scaille, and Tammy Morshed for helpful comments in the preparation of this manuscript.

References


