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c-Cbl: A regulator of T cell receptor-mediated signalling

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Summary The 120-kDa protein product of the c-Cbl proto-oncogene is a ubiquitously expressed cytoplasmic protein that is especially abundant in the thymus, indicating an important role for Cbl in thymic signalling. c-Cbl possesses a highly conserved N-terminal phosphotyrosine binding domain, a C3HC4 RING finger motif, multiple proline-rich motifs, and a number of potential tyrosine phosphorylation sites. Cbl is an early and prominent substrate of protein tyrosine kinases following stimulation of a variety of cell surface receptors, and forms constitutive and inducible associations with a wide range of signalling intermediates. Genetic studies of the Cbl homologue Sli-1 in Caenorhabditis elegans predicted a role for Cbl as a negative regulator of protein tyrosine kinase-mediated signalling pathways. Numerous studies have now shown that expression of Cbl and its oncogenic variants can indeed modulate signalling from activated protein tyrosine kinases. The present review highlights some of the recent developments in our understanding of Cbl function, with particular reference to its participation and possible roles in TCR-mediated signalling.

Key words: Cbl, protein tyrosine kinases, signal transduction, T cell receptor.

Introduction

Biochemical signalling pathways play a crucial role in directing cellular fate and function. The communication route by which a signal received from the cell’s external environment is relayed within the cell to generate the appropriate response is, by necessity, a tightly controlled process. Deregulation of these networked processes can lead to aberrant signalling and cellular transformation. Indeed many key signalling intermediates have been identified through their ability to induce cellular transformation when inappropriately expressed or expressed in an altered form. One such molecule is the protein product of the c-Cbl proto-oncogene.

The c-Cbl (Casitas B-lineage lymphoma) gene was first identified as the cellular homologue of a virally transduced transforming gene found in the murine Cas NS-1 retrovirus.1 Introduction of Cas NS-1 induces pre-B cell lymphomas and myeloid leukaemias in mice and transforms fibroblast cell lines.1 The transforming gene, v-Cbl, was shown to encode a markedly truncated form of c-Cbl, expressing only the N-terminal 355 amino acids (aa) of the 906aa full length cellular protein. c-Cbl encodes a 120-kDa cytoplasmic protein that is expressed ubiquitously, with highest expression levels in the testes and cells of haemopoietic lineage.2

Interest in Cbl has escalated in recent years, with a growing body of evidence that Cbl is not only an early and prominent substrate following activation of cell surface receptor and receptor-associated tyrosine kinases, but that it may also play a regulatory role in modulating the signals relayed through these pathways. The present review summarizes recent studies examining the function and protein interactions of the c-Cbl, with particular emphasis on Cbl’s role in TCR-mediated signalling.

The Cbl protein family

c-Cbl is a large multidomain protein with no known catalytic activity but it does possess several distinctive features, as shown in Fig. 1. The N-terminal half of the Cbl protein encompassed by v-Cbl is highly basic in charge and was recently demonstrated to contain a novel phosphotyrosine binding (PTB) domain.3–5 The C-terminal portion is characterized by a large proline-rich domain providing multiple binding sites for a range of SH3 domain-containing proteins, and is separated from the PTB domain by a C3HC4 zinc RING finger motif. A ubiquitination recognition sequence and putative leucine zipper motif is also found at the very C terminus of the Cbl protein.5,6 The C-terminal tail also contains multiple tyrosine residues which, upon phosphorylation, form binding sites for SH2 domain-containing proteins.

A number of Cbl homologues have now been identified in mammals and invertebrates. The Cbl family to date consists of mammalian c-Cbl and the closely related Cbl-b, Drosophila Cbl (D-Cbl) and the C. elegans Sli-1 (Fig. 1a).7–10 Cbl-b was isolated from a human breast cancer cDNA library and is also widely expressed, although c-Cbl is the more abundant protein in haemopoietic tissues.5,7 All four Cbl proteins demonstrate a remarkably high degree of homology over the N-terminal region which Bustelo et al. term the ‘Cbl homology’ or CH domain,11 but diverge almost immediately after the RING finger. This suggests that the N-terminal region containing the PTB domain mediates

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an important evolutionarily conserved role for Cbl proteins. This is further shown by the fact that while Cbl-b and Sli-1 still retain a number of proline-rich SH3-binding motifs, D-Cbl consists entirely of the CH/PTB domain and the RING finger motif.

Studies of Cbl function have extensively utilized two oncogenic Cbl mutants which arose in vivo; the C-terminally truncated v-Cbl oncogene and a mutant form of Cbl isolated from the 70Z/3 pre-B lymphoma line. 70Z-Cbl oncogenicity was attributed to a 17aa deletion at the N terminus of the RING finger motif arising from a splice acceptor site mutation.12 The two mutants differ in that unlike v-Cbl, 70Z-Cbl retains almost all the characteristic features of c-Cbl, in particular the major protein-binding domains (Fig. 1b). Furthermore, while v-Cbl is not detectably phosphorylated, 70Z-Cbl exhibits enhanced tyrosine phosphorylation relative to wild-type c-Cbl.12–14 Thus v-Cbl is often viewed as having a dominant negative effect compared with c-Cbl for protein binding sites, while 70Z-Cbl functions in a positive manner, being able to interact with Cbl-binding partners but no longer exerting a negative regulatory effect.

**Cbl as a negative regulator of tyrosine kinase-mediated signalling pathways**

The constitutive tyrosine phosphorylation of 70Z-Cbl implied that interactions with, and subsequent phosphorylation by, activated tyrosine kinases might be a key to Cbl function. A role for c-Cbl in intracellular signalling pathways involving tyrosine kinases was first demonstrated when it was identified as the 120-kDa major phosphoprotein in activated T cells.15 Since then we and many others have shown that Cbl is rapidly and prominently tyrosine phosphorylated following activation of a wide range of cell surface receptors (Table 1). These include the T cell and B cell antigen receptors,15–19 the CD19, CD38, CD16 and CD5 lymphocyte receptors,20–24 Fc receptors such as FcεRI and FcγRI,25–27 growth factor receptors for the epidermal growth factor (EGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), nerve growth factor (NGF), colony stimulating factor (CSF-1), c-Kit/Steel factor and prolactin,4,28–33 cytokine receptors for granulocyte/macrophage colony stimulating factor (GM-CSF), erythropoietin, thrombopoietin,44 IL-2, IL-3, IL-4, and interferon-α as well as integrin receptors.41,42 Cbl phosphorylation following stimulation of the insulin receptor has also been shown, although this appears to be restricted to differentiated adipocytes.41,42 Constitutive Cbl tyrosine phosphorylation is also observed in cells transformed by activated Src and Abl,12 indicating a pivotal role for Cbl in signal transduction mediated by both receptor and non-receptor tyrosine kinases.

Evidence that Cbl plays an important role in regulating protein tyrosine kinase (PTK) signalling pathways originated from genetic studies of the Cbl homologue Sli-1 in *C. elegans*. Yoon et al. and Jongeward et al. found that loss or mutation of Sli-1 was able to restore signalling through a weakly active EGF receptor homologue (Let-23).10,43 Furthermore, introducing additional copies of the Sli-1 gene had a suppressive effect on Let-23 signalling.43 These experiments demonstrated that Sli-1 acts as a negative regulator of the Let-23 receptor, exerting its effects at the level of the receptor and the *C. elegans* Grb2 homologue Sem5. Such findings are consistent with mammalian studies that place Cbl at an early point in cell signalling pathways involving PTK.

**Figure 1** Major features of Cbl proteins. (a) Diagrammatic representation of major features of mammalian c-Cbl, Cbl-b, *Caenorhabditis elegans* Sli-1 and *Drosophila* Cbl (D-Cbl). Shown are the phosphotyrosine binding (PTB) domain (grey) contained within the N-terminal basic region (+), the C_H3 RING finger, potential SH3-binding proline-rich motifs (dark shaded blocks), and the C-terminal ubiquitin binding sequence and putative leucine zipper (Ub/LZ). Percentages indicate the percentage amino acid homology of the highly conserved N-terminal region containing the PTB and RING finger domains of the various Cbl proteins relative to c-Cbl. Numbers in parentheses indicate overall amino acid homology over the full length protein. (b) Diagrammatic representation showing the regions deleted from the oncogenic 70Z-Cbl and v-Cbl proteins.

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An increasing number of studies are now providing supportive evidence that Cbl acts as a negative regulator of tyrosine-kinase-mediated signalling in mammalian cells. Overexpression of c-Cbl suppresses FcεRI-induced Syk kinase activity and serotonin release from mast cells, and decreases Ras-dependent Erk2 and AP1 activation in activated T cells. 70Z-Cbl but not v-Cbl or c-Cbl induces transcriptional activation of the nuclear factor of activated T cells (NFAT), which is abrogated by expression of a dominant negative Ras, implicating 70Z-Cbl in the Ras pathway. Overexpression of Cbl-b was also shown to inhibit Vav-mediated c-JNK activation, acting specifically at the level of c-JNK with no effect on the upstream MEKK activator. Thus Cbl protein family members may share a conserved function as negative regulators. Importantly in all these studies, the effect of Cbl proteins are mediated from an initiating signal, consistent with genetic studies in C. elegans which showed that the effects of Sli-1 were still signal dependent. However, it should be noted that a negative effect is not always observed; c-Cbl expression has been shown to enhance the Ras-independent anti-apoptotic activity of IL-4 in B cells, an effect dependent on its interaction with the phosphatidylinositol-3 kinase (PI-3K).

Cbl in T cells

Cbl as a complex adaptor protein

c-Cbl is particularly abundant in the thymus, and is one of the earliest and most prominent tyrosine phosphorylated substrates detected following T cell activation, indicating an important role for Cbl in thymic signalling. Consistent with a probable role as a regulatory protein, Cbl interacts with a number of key signalling intermediates, some of which are shown in Fig. 2. These associations are primarily mediated by Src homology (SH)3, SH2 or Cbl PTB domain interactions. Cbl associates constitutively with the adaptor proteins Grb2 and Nck, and this interaction occurs through binding of their SH3 domains to proline-rich motifs in Cbl. Recently the 88-kDa SH3 domain containing Cbl-associated protein (CAP) was cloned from 3T3-L1 adipocytes using the yeast two-hybrid system and shown to bind constitutively to Cbl proline-rich motifs. Growth factor or antigen receptor stimulation induces complexes between tyrosine phosphorylated Cbl and SH2 domains of the Crk adaptor proteins, the p85 regulatory subunit phosphatidylinositol-3 kinase (PI-3K), and the guanine nucleotide exchange protein Vav. Other proteins such as the Fyn non-receptor tyrosine kinase, associate constitutively with Cbl through an SH3 domain but this interaction increases following Cbl tyrosine phosphorylation through additional SH2 domain binding. Recently it was shown that the N-terminal region of Cbl contains a novel PTB domain that binds directly to the phosphorylated ZAP-70 non-receptor tyrosine kinase, EGFr receptor and PDGF receptor. Cbl PTB binding ability is abolished by a G306E mutation corresponding to a loss-of-function mutation in Sli-1 which abolishes PTB ability and abrogates v-Cbl transformation in c-Cbl. SH3 domains of adaptor proteins such as Grb2 and Nck, and the Src kinases, Fyn and Src bind to proline-rich motifs of Cbl. Also shown is a serine-rich region (S) which recruits 14-3-3 proteins following TCR stimulation. The three major tyrosine phosphorylation sites of Cbl are indicated, and these recruit the SH2 domain-containing proteins Vav, CrkL and PI-3K as shown.

Cbl and Grb2

The Cbl/Grb2 complex was the first to be identified in T cells, when Cbl was shown to be the major 120-kDa phosphoprotein associated with Grb2 in activated T cells. This interaction occurs constitutively and is mediated by the N-terminal SH3 domain of Grb2 binding to several proline-rich motifs found between amino acids 481–563 of c-Cbl. The Cbl/Grb2 association appears to be an important one because between one-third to half of the total Cbl protein has been reported to associate with Grb2 in
unstimulated T cells. Both Cbl and the Ras guanine nucleotide exchange factor, Sos, preferentially bind the N-terminal SH3 domain of Grb2. Not surprisingly then, Grb2/Cbl and Grb2/Sos complexes are found to be distinct in T cells. Interestingly, T cell stimulation initiates dissociation of the Cbl/Grb2 complex which may be due to decreased affinity of the Grb2 SH3 domain for activated Cbl. Rellahan et al. showed that the inducible dissociation of Cbl from Grb2 corresponds to an induced association of Grb2 with Sos. In growth factor signalling, Grb2 coupling of receptors to Sos is an important step in activating the Ras pathway. It is possible then that Cbl may regulate Ras activation in T cells by sequestering Grb2 from binding Sos prior to T cell activation, although this does not seem likely given that only an estimated 3% of total Grb2 is associated with Cbl.

**Cbl and Fyn**

Significant Cbl tyrosine phosphorylation can be detected within 1 min of stimulation, demonstrating a proximal role for Cbl in TCR signalling. Cbl phosphorylation is abolished in Fyn−/− thymocytes but can still be induced in Lck-deficient JCam1.6 T cells, and Lck transfected into COS cells phosphorylates Cbl poorly in comparison to Fyn. Furthermore, Cbl demonstrates much stronger in vivo association with Fyn than Lck. These results implicate Fyn as the major tyrosine kinase responsible for phosphorylating Cbl in T cells, although other kinases such as Yes and Syk may also contribute. The major sites of TCR-induced tyrosine phosphorylation have recently been mapped to Y700, Y731, and Y774 in the C-terminal tail of c-Cbl. Experiments using the yeast two-hybrid system and truncated c-Cbl mutants indicated that aa552–614 were responsible for constitutive binding to Fyn, presumably due to the Fyn SH3 domain binding proline-rich motifs within this region of Cbl. Following TCR stimulation, the Fyn SH2 domain provides an additional means of association, although the tyrosine residue recognized has not yet been determined.

**Cbl and Crk**

Following T cell activation, Cbl rapidly complexes with the CrkL adaptor protein that binds via its SH2 domain to phosphorylated Y774 (pY774). Virtually all the tyrosine phosphorylated Cbl protein can be co-immunoprecipitated with CrkL, even though this represents only a portion of the total Cbl protein in the cell. It is noteworthy that we previously showed that Cbl Y700 and Y774 were two major sites phosphorylated by activated Ab1 kinases and that this induces a prominent association with the CrkL adaptor protein through its SH2 domain. Crk protein association also accounts for a significant portion of tyrosine phosphorylated Cbl in EGF-stimulated cells. These findings indicate that phosphorylation of Y700 and Y774 residues and Cbl’s interaction with CrkL are important mediators of Cbl function. CrkL is constitutively bound via its N-terminal SH3 domain to C3G, a nucleotide exchange factor for the Rac/Rho family of small G proteins, including the Ras-related protein, Rap1. Interestingly Rap1 is thought to negatively regulate Ras signalling by competing for the same downstream effectors such as Raf-1. A TCR-inducible association between Cbl and C3G was detected in Cbl-overexpressing Jurkat T cells demonstrating that a trimeric complex of Cbl-CrkL-C3G can be formed in vivo. Evidence suggesting that such a ternary complex may have functional significance comes from a study of anergic T cells, in which increased Fyn kinase activity corresponds to constitutively Cbl tyrosine phosphorylation and formation of a Cbl-CrkL-C3G complex, and this is associated with constitutively activated Rap1-GTP and a block in the Ras pathway. In non-obese diabetic (NOD) mice, hyporesponsiveness to T cell activation due to impaired membrane targeting of the Grb2/Sos complex was shown to be associated with enhanced Fyn kinase activity and differential activation of the Fyn-TCR-Cbl pathway. Thus in T cells, Crk and Grb2 appear to preferentially couple to distinct guanine nucleotide exchange factors that regulate Rap1 and Ras, respectively, and the relative associations of Cbl with both these adaptor proteins may be involved in the balance between these two pathways.

**Cbl and Vav**

Cbl association with a third guanine nucleotide exchange factor, Vav, has also been demonstrated which is mediated by the Vav SH2 domain binding to phosphorylated Y700 of c-Cbl. Vav has been implicated in Ras activation in T cells, and synergizes with SLP-76 to augment NFAT activation and IL-2 transcription. A role for Cbl in regulating these Ras-dependent pathways has previously been inferred from findings that 70Z-Cbl constitutively activates NFAT activation and that c-Cbl overexpression can suppress AP-1 activation. However, whether these effects of Cbl are mediated through regulation of Vav activity is yet to be determined.

**Cbl and PI-3K**

Another prominent interaction in activated T cells is the induced association between c-Cbl and the p85 regulatory subunit of the PI-3 lipid kinase (PI-3K) through binding of p85 SH2 domains to the phosphorylated Y731EAM motif in c-Cbl. A small fraction of c-Cbl may also be constitutively associated with p85 via its SH3 domain, although evidence for this interaction is only inferred by in vitro binding studies. Hartley et al. showed that c-Cbl specifically recognizes the β isofrom of p85 and that Cbl is the only major phosphoprotein associated with p85 in T cells. T cell receptor ligation stimulates PI-3K kinase activity, and a disproportionately large increase in this activity is detected in Cbl immunoprecipitates. This increase is particularly concentrated in the membrane and insoluble particulate fractions, where a small but highly tyrosine-phosphorylated fraction of Cbl is translocated following T cell stimulation. Indeed, membrane-associated Cbl was found to contain 20-fold more p85 than cytosolic Cbl. Cbl may thus serve to recruit active p85 to the plasma mem-
brane, because PI-3K does not directly associate with the TCR/CD3 complex.

PI-3K does not appear to be involved in mediating mitogenic responses in T cells because this response is insensitive to the effects of wortmannin, an inhibitor of PI-3K activity. Consistent with that, 70Z-Cbl enhancement of the Ras-dependent NFAT activation was shown to be independent of its ability to bind PI-3K. Rather PI-3K activity is thought to be involved in the activation of integrin binding to fibronectin in T cells. Ojaniemiet al. report induced Cbl/PI-3K association following activation of integrin signalling pathways, and we have found that expression of different Cbl mutants can greatly alter cell shape and cytoskeletal rearrangement in response to PDGF stimulation, effects that can be inhibited by wortmannin (R. Scaife and W. Langdon, unpubl. data). These results strongly suggest that Cbl and PI-3K may function in signal transduction pathways involved in integrin activation and cell adhesion. PI-3K has also been implicated in down-regulation of the PDGF receptor. It will be of interest to determine whether the Cbl/PI-3K complex also affects TCR trafficking given Cbl’s recently described involvement in growth factor receptor internalization and degradation (G. Levkowitz and Y. Yarden, pers. comm., 1998).

**Cbl and ZAP-70**

Fournel et al. first identified c-Cbl as the 120-kDa phosphoprotein associated with ZAP-70 tyrosine kinase in activated T lymphocytes. While they showed that the interaction was phosphotyrosine dependent, they were unable to demonstrate Cbl binding by the ZAP-70 SH2 domains. Subsequently it was found that Cbl binding to ZAP-70 is direct and mediated by a novel phosphotyrosine binding (PTB) domain encompassed by the N-terminal 355aa of c-Cbl. Cbl PTB binding is now known to also contribute to its interaction with the EGF and PDGF receptors.

Recently the screening of a phosphopeptide library identified the sequence D(N/D)XpY (where X is any amino acid) as the preferred recognition motif for the Cbl PTB domain. The three major phosphorylation sites within ZAP-70 have been mapped to Y292, Y492 and Y493. Phosphorylation of Y493 by Lck is required to activate ZAP-70 kinase activity which then promotes phosphorylation of other sites, including Y292 and Y492. Both Y292 and Y492 are considered to have negative regulatory roles in ZAP-70 because mutation of either of these residues to phenylalanine results in enhanced tyrosine phosphorylation of cellular proteins and increased calcium mobilization following T cell activation. However, only the Y492F ZAP-70 displays hyperresponsive TCR signal-dependent kinase activity. Y292 is believed to exert its effects indirectly on ZAP-70 by binding a negative regulatory protein. It is interesting, therefore, that while Y292, Y492 and Y493 all lie within sequences conforming to the Cbl PTB recognition motif, peptide competition and GST pull-down assays showed that only Y292 is recognized by c-Cbl and required for in vivo Cbl/ZAP-70 interaction. This interaction is completely ablated by the G306E mutation which abrogates Cbl PTB binding ability as well as c-Cbl transformation. Peptides closely matching the Y292 negative regulatory sequence are represented in several other proteins to which c-Cbl binds, including the EGF receptor, Syk, and PDGF receptor, suggesting a potential general mechanism for c-Cbl’s regulation of protein tyrosine kinases. These findings are consistent with studies in C. elegans, predicting a conserved negative regulatory role for Cbl in tyrosine kinase-mediated signalling which is dependent on its conserved N-terminal domain.

**Deregulated thymocyte signalling in c-Cbl-deficient mice**

The generation of a c-Cbl-deficient mouse has also provided additional evidence to support a negative regulatory role for Cbl in TCR-mediated signalling. Loss of c-Cbl is characterized by hyperplastic changes in mammatory and lymphoid tissues. Mammary hyperplasia and increased ductal branching may reflect enhanced growth factor responsiveness to EGF family ligands in the absence of c-Cbl, given Sli-1’s proposed role in attenuating EGF receptor signalling in C. elegans and c-Cbl’s interaction with the EGF receptor in mammalian cells. Spleens from c-Cbl−/− mice show abundant extramedullary haemopoiesis, increased numbers of megakaryocytes and disrupted internal architecture. While c-Cbl−/− mice display the complete developmental repertoire of thymocytes, there is a significant increase in the cell surface expression of TCRβ and CD3ε receptors. Collectively, these hyperplastic changes are further evidence of Cbl’s normal involvement in placing regulatory restraints on cellular growth pathways.

Analysis of TCR signalling in c-Cbl−/− thymocytes revealed a marked hyperphosphorylation of cellular substrates following TCR stimulation (Fig. 3a). Among the major phosphoproteins detected were those with molecular weights of 36–38 kDa, 70 kDa, 75 kDa and 80 kDa. The 70-kDa phosphoprotein was identified as the ZAP-70 tyrosine kinase and immunoblotting showed that other phosphoproteins co-migrate with SLP-76, the Lck-binding phosphoprotein associated with ZAP-70 tyrosine kinase in activated T lymphocytes. Fournel et al. first identified c-Cbl as the 120-kDa phosphoprotein associated with ZAP-70 tyrosine kinase in activated T lymphocytes. While they showed that the interaction was phosphotyrosine dependent, they were unable to demonstrate Cbl binding by the ZAP-70 SH2 domains. Subsequently it was found that Cbl binding to ZAP-70 is direct and mediated by a novel phosphotyrosine binding (PTB) domain encompassed by the N-terminal 355aa of c-Cbl. Cbl PTB binding is now known to also contribute to its interaction with the EGF and PDGF receptors.

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Analysis of TCR signalling in c-Cbl−/− thymocytes revealed a marked hyperphosphorylation of cellular substrates following TCR stimulation (Fig. 3a). Among the major phosphoproteins detected were those with molecular weights of 36–38 kDa, 70 kDa, 75 kDa and 80 kDa. The 70-kDa phosphoprotein was identified as the ZAP-70 tyrosine kinase and immunoblotting showed that other phosphoproteins co-migrate with SLP-76, the Lck-binding protein HS1 and pp36–38 LAT, which are known substrates of ZAP-70. This suggested that loss of c-Cbl can negatively regulate the ZAP-70 tyrosine kinase.

The T cell antigen receptor (TCR)/CD3 complex is a multichain aggregate consisting of a TCR α/β or γ/δ binding heterodimer that is non-covalently associated with the signal transducing subunit comprising a TCR ζ-ζ homodimer, and CD3ε-γ and CD3ε-δ paired chains. Engagement of the TCR induces tyrosine phosphorylation of cellular proteins, elevation of intracellular calcium, activation of lipid-dependent kinases, and activation of Ras. Downstream effects of this include induction of IL-2 transcription through NFAT and AP-1 activation, and cellular proliferation. While the TCR/CD3 complex possesses no intrinsic kinase activity, it is associated with two families of PTK; the p56Lck and p59Fyn Src family PTK, and the ZAP-70/Syk PTK family. Lck is constitutively associated with the CD4 or CD8 co-receptor molecules while Fyn binds to non-phosphorylated immunoreceptor tyrosine-based motifs (ITAM) which are present in multiple copies within the cytoplasmic tails of the CD3 and TCRζ chains. Following TCR ligation, the Src family PTK
induce phosphorylation of ITAM and this creates multiple binding sites for SH2 domain-containing proteins such as phospholipase C\(_c\) (PLC\(_c\)) and ZAP-70. Recruitment of these proteins to the TCR complex is necessary for their tyrosine phosphorylation and enzymatic activation, and is a key step in transducing the TCR-mediated signal.\(^8\) In normal murine thymocytes, ligation of the TCR/CD3 complex induces activation of the CD3\(\varepsilon\)-associated Fyn kinase, while activation of Lck requires coaggregation of the TCR/CD3 complex with CD4 or CD8 co-receptors.\(^7\)\(^2\)\(^9\) The striking feature observed in \(c-Cbl\) thymocyte signalling was the uncoupling of ZAP-70 tyrosine phosphorylation from a requirement for Lck activation through CD4 cross-linking with the TCR complex. (Fig. 3). Hyperphosphorylation of ZAP-70 and its substrates was detected in the absence of overt Lck kinase activation following stimulation of the TCR/CD3 complex alone, where such events are not normally observed (Fig. 3).\(^7\)^\(^2\)\(^9\) As expected, CD4 cross-linking induced Lck kinase activation (Fig. 3b) and comparable ZAP-70 tyrosine phosphorylation in both normal and \(c-Cbl\) thymocytes, although \(c-Cbl\) thymocytes consistently show a slightly greater phosphorylation. Even then, a disproportionately large increase in the phosphorylation of the ZAP-70 substrates SLP-76 and LAT was detected in \(c-Cbl\) cells relative to wild-type cells (Fig. 3a). Time-course analysis showed that hyperphosphorylation of substrates in \(c-Cbl\) thymocytes was not due to inability to be dephosphorylated.\(^4\)\(^3\) Part, but not all, of the hyperresponsiveness of \(c-Cbl\) thymocytes to receptor occupation may be explained by a moderate increase in TCR\(\beta\) and CD3 surface receptors. However, the selective and qualitative increase in substrate phosphorylation and the difference in ZAP-70 SLP-76 phosphorylation ratio are indicators that loss of Cbl does remove some regulatory restraint. These results indicate that loss of Cbl results in the inappropriate activation of ZAP-70 kinase activity via CD3 that bypasses its requirement for Lck activation.

Given the marked up-regulation of proximal events in TCR signalling, it was perhaps surprising that no significant differences were observed between wild-type and \(c-Cbl\) thymocytes further downstream. \(c-Cbl\) mice demonstrated normal antigenic responses and \(in vitro\) proliferation in response to TCR cross-linking. Increased activation of the extracellular signal-regulated kinase (ERK) was observed in \(c-Cbl\) thymocytes, however, this was inducible and transient rather than constitutive.\(^4\)\(^3\) One possibility is that functional redundancy with the related Cbl-b may allow cells to compensate for the loss of Cbl, and the availability of Cbl-b-deficient mice in the future will allow this possibility to be addressed. Another possibility to consider is that Cbl functions in cooperation with other negative regulatory proteins, and that loss of Cbl compromises but does not fully ablate the regulatory restraints operating in the various signalling pathways. This is the case in \(C.\) \(elegans\), where mutation of \(Sli-1\) alone does not produce any observable phenotypic changes but requires additional mutation to the \(Let-23\) receptor or early signalling components for its negative regulatory effects to be revealed.\(^4\)\(^3\)

**Conclusion**

There is now a compelling body of evidence that Cbl is a major player in cell signalling pathways mediated by pro-
tein tyrosine kinases, and that one of its conserved functions is to negatively regulate such pathways. Abundant expression of Cbl in the thymus suggested an important role for Cbl in TCR-mediated signalling. This has been borne out by studies showing Cbl as a prominent early substrate of TCR-activated kinases, as well as an adaptor molecule centrally positioned to interact with key signalling intermediates and to modulate the various pathways activated upon TCR stimulation (Fig. 4). Suppression of Syk and AP-1 activation by c-Cbl and the deregulated hyperphosphorylation of protein substrates in c-Cbl-deficient thymocytes further demonstrate the potential of Cbl to regulate pathways in thymic signalling. Specifically Cbl appears to be able to negatively regulate signalling by the Syk/ZAP-70 family of protein tyrosine kinases.

The question remains, then: how does Cbl exert its negative regulatory effects, and what role does the evolutionarily conserved PTB domain play? One possibility is that Cbl is able to directly modulate tyrosine kinase activity. We showed that expression of 70Z-Cbl greatly increased EGF receptor kinase activity.\(^4\) Ota and Samelson found that the suppressive effect of c-Cbl overexpression in FcεRI-stimulated mast cells was specific for the Syk tyrosine kinase with no apparent inhibition on Lyn, the Src family kinase acting directly upstream of Syk.\(^5\) Similarly Cbl may directly impact on ZAP-70 kinase activity in activated thymocytes, and its unavailability to bind ZAP-70 may explain the removal of normal regulatory restraints on ZAP-70 kinase activation and substrate phosphorylation in c-Cbl−/− cells. Cbl binds ZAP-70 through Y292, a known negative regulatory site in ZAP-70. However, while mutation of Y292 results in signal-dependent hyperphosphorylation of cellular substrates, this effect appears to be independent of any change to ZAP-70 kinase activity,\(^6\) suggesting that Cbl may not affect intrinsic ZAP-70 kinase activity.

Alternatively Cbl may affect ZAP-70 signalling by modulating ZAP-70 substrate affinity, or by altering the subcellular localization of ZAP-70 and its substrate proteins. A role for Cbl in regulating receptor down-regulation has also been proposed, with demonstrated involvement in ubiquitination and subsequent degradation and endocytic sorting of the CSF-1 and EGF receptors, respectively, (G. Levkowitz and Y. Yarden, pers. comm., 1998).\(^7\) c-Cbl−/− thymocytes display increased numbers of TCRβ and CD3 surface receptors, but this does not appear to be due to deficiencies in the ability of the receptor to be internalized (D. Bowtell, pers. comm., 1998).\(^8\) Nonetheless these results suggest that Cbl may function to regulate receptor trafficking and hence alter cellular responsiveness to growth factor or antigen stimulation.

Beginning with predictions from genetic studies in *C. elegans*, we now have ample evidence supporting a conserved role for Cbl proteins as modulators of PTK signalling in mammalian systems. The central role of tyrosine kinases in intracellular signalling pathways controlling cell growth and differentiation highlights the importance of and interest in promoting further studies to pinpoint the biochemical mechanisms by which this unique family of proteins exert their negative regulatory effects.

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**Figure 4** Cbl occupies a potentially pivotal position in TCR-mediated signalling pathways. The TCR activation by antigen binding activates the Fyn tyrosine kinase which phosphorylates Cbl and promotes its association with Vav, CrkL, p85 proteins, and induces its dissociation from Grb2. Serine phosphorylation of Cbl induces binding to 14-3-3 proteins. In normal thymocytes, cross-linking of the CD4 co-receptor with the TCR/CD3 complex is required to activate the Lck tyrosine kinase which then phosphorylates ZAP-70 and activates its kinase activity, leading to phosphorylation of downstream substrates including LAT and SLP-76. Evidence indicates that Cbl may directly negatively regulate the ZAP-70 tyrosine kinase (bold arrow). Each of these Cbl-binding proteins has been shown to feed into pathways regulating TCR-mediated responses, some of which are shown here, demonstrating Cbl’s central position in TCR signalling.

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