Cbl-mediated ubiquitinylation and negative regulation of Vav

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ABSTRACT

The Cbl ubiquitin ligase has emerged as a negative regulator of receptor and non-receptor tyrosine kinases. Cbl is known to associate with the proto-oncogene product Vav, a hematopoietic-restricted Rac guanine nucleotide exchange factor, but the consequences of this interaction remain to be elucidated. Using immortalized T cell lines from Cbl$^{+/}$ and Cbl$^{-/-}$ mice, and transfection analyses in 293T cells, we demonstrate that Vav undergoes Cbl-dependent ubiquitinylation under conditions that promote Cbl and Vav phosphorylation. Interaction with Cbl also induced the loss of phosphorylated Vav. In addition, we show that an activated Vav mutant (Vav-Y174F) is more sensitive to Cbl-dependent ubiquitinylation. We demonstrate that the Cbl-dependent ubiquitinylation of Vav requires Cbl/Vav association through phosphorylated Y700 on Cbl, and also requires an intact Cbl RING finger domain. Finally, using transfection analyses in the Jurkat T cell line, we show that Cbl, but not its ubiquitin ligase mutant, can inhibit Vav-dependent signaling. Thus, our findings strongly support the role of Cbl, via its ubiquitin ligase activity, as a negative regulator of activated Vav.
INTRODUCTION

Activation of protein tyrosine kinases (PTKs) represents a fundamental mechanism by which higher eukaryotic cells respond to changes in the extracellular milieu. Less is known, however, about the mechanisms that fine tune the level of PTK activation and their downstream signaling pathways; such regulatory controls are essential to prevent pathological manifestations of PTK activation, such as cellular transformation or autoimmunity. Recent studies by us and others have revealed that the proto-oncogene product Cbl and the related family members Cbl-b and Cbl-c function as critical negative regulators of PTKs (1-8).

The PTK regulatory function of Cbl involves its ability to function as a ubiquitin ligase (E3) and thereby to target activated PTKs for ubiquitinylation. The N-terminal region of Cbl, composed of a four-helical bundle, a calcium-binding EF hand motif and an incomplete Src-homology 2 (SH2) domain (1), functions as a Tyrosine Kinase-Binding (TKB) domain by directly interacting with specific autophosphorylation sites in activated PTKs, such as growth factor receptor tyrosine kinases (RTKs) and ZAP70/Syk PTKs (2-5). Alternatively, the proline-rich region in the middle part of Cbl protein can mediate its direct interaction with SH3 domains of Src-family kinases (6). The RING finger (RF) domain of Cbl, located between the TKB domain and the proline-rich region, binds to ubiquitin conjugation enzymes (Ubcs), such as UbcH5B and UbcH7 (7-11), providing a basis for the ubiquitin ligase activity of Cbl towards associated PTKs. The ubiquitin moiety attached to activated RTKs functions as a lysosomal sorting signal,
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resulting in enhanced lysosomal degradation (12-15). Ubiquitinylation of Syk and Src-family kinases appears to target them for enhanced proteasomal degradation (14,16-19). Thus, Cbl-mediated ubiquitinylation provides a mechanism to eliminate the activated pools of PTKs via protein degradation, complementing dephosphorylation and other regulatory processes.

Studies with mammalian Cbl-b and Cbl-c, as well as C. elegans and Drosophila Cbl homologues, have demonstrated the conservation of this mechanism (20,21). Reflecting a critical role of Cbl-family proteins as negative regulators of PTK signaling, the Cbl-deficient mice exhibit hypercellular lymphoid organs and increased branching morphogenesis of the mammary gland, whereas the Cbl-b deficient mice demonstrate fulminant autoimmune disease either spontaneously or upon immunization with autoantigens (22-24). Importantly, Cbl/Cbl-b double knockout mice are embryonic lethal (25).

While the functional roles of the association of Cbl with PTKs have begun to be elucidated, the role of Cbl interactions with other components of PTK signaling has remained less clear. Cbl functions as a major substrate of phosphorylation by various PTKs with which it interacts (6,8), resulting in stimulation-induced association of Cbl with SH2 domain-containing signaling proteins. Three well-characterized tyrosine phosphorylation sites in the C-terminal part of Cbl mediate its association with Vav (Y700), the p85 subunit of phosphatidylinositol-3 kinase (Y731), and the adaptor proteins of the Crk family (Y774) (4,6,8,26-28). The potential functional consequences of these
associations are of great interest. Two fundamentally opposite roles, which are not necessarily mutually exclusive, have been suggested. One view is that phosphorylated Cbl serves as a scaffold to assemble signaling complexes, thus facilitating PI 3-kinase-, Vav- or Crk-dependent cellular responses, such as cell survival and migration (20).

Given the recent appreciation of the evolutionarily conserved function of Cbl proteins as ubiquitin ligases, we and others have suggested that Cbl may also target non-kinase signaling proteins for ubiquitinylation, which in turn may either target these proteins for degradation or regulate their function independently of degradation (20,29). Notably, Cbl-b was shown to constitutively interact with the p85 subunit of PI 3-kinase and induce PI 3-kinase ubiquitinylation, although a change in protein levels was not observed nor did proteasome inhibitors increase the levels of ubiquitin-tagged PI 3-kinase. In conjunction with the inhibitory effect of PI 3-kinase inhibitors on the hyperactive phenotype of Cbl- b<sup>−/−</sup> T cells, these findings led the authors to suggest that Cbl-b-dependent ubiquitinylation negatively regulates the PI 3-kinase activity in a degradation-independent manner (30); however, it remains possible that the inability of the investigators to detect an effect of Cbl on PI 3-kinase levels may simply reflect a small size of the Cbl-associated PI 3-kinase pool. Thus there is a clear need to further elucidate the biochemical and functional consequences of the association of Cbl with non-PTKs targets. In this study, we have focused on the role of the association of Cbl with Vav.

Vav is a hematopoietic-restricted member of a family (Vav, Vav2, and Vav3) of guanine nucleotide exchange factors (GEFs) for the Rho family of small GTPases, especially Rac and Rho (31-34). Vav proteins contain a calponin-homology domain, an
acidic region, a plekstrin-homology (PH) domain, a Dbl-homology (DH) domain characteristic of all Rho family GEFs, a zinc finger domain, and two SH3 domains flanking a single SH2 region (31). Vav-mediated Rac activation plays an important role in antigen receptor-induced cytoskeletal reorganization, and activation of stress-activated protein kinases and several important transcription factors, such as the nuclear factor of activated T cells (NF-AT) and NF-κB (35-38). Several biochemical and genetic studies have established a critical role for Vav in the development of T cells and their activation though the T cell antigen receptor (TCR). Notably, Vav-deficient mice show a lack of IL-2 gene transcription in response to TCR triggering and impaired positive selection of thymocytes (39-42). Among over forty known human Rho/Rac GEFs, the Vav family is distinct in that the activity of these proteins is directly modulated by tyrosine phosphorylation (35). Vav is tyrosine phosphorylated in response to stimulation of the T and B cell antigen receptors in a Syk/ZAP70- and Src-family PTK-dependent manner (43-46). While phosphorylation of several tyrosine residues regulates Vav activity, that of Tyr174 in the acidic region is quite critical, as it relieves the DH domain from the autoinhibition imposed by its intramolecular association with the N-terminal region (47). Indeed, a Y→F mutation at Tyr174 converts Vav into an oncogene, and enhances Vav-mediated cytoskeletal reorganization as well as JNK and NF-AT activation (48,49).

A number of recent studies suggest a potential role for Cbl in regulating Vav. Vav was shown to interact with Cbl in both thymocytes and peripheral T cells upon stimulation through the TCR (27). Phosphopeptide competition experiments suggested that the Cbl/Vav association was mediated by the Vav SH2 domain binding to
phosphorylated Y700 on Cbl (27). Notably, T cells from Cbl-b deficient mice showed enhanced Vav phosphorylation and TCR clustering upon TCR stimulation (23,24,50), while Cbl-b deficiency restored the defective TCR clustering observed in Vav^{+/−} T cells (24). However, studies directly establishing a role for Cbl family proteins in the regulation of Vav are lacking, and it is unknown whether the Cbl ubiquitin ligase activity is involved in such regulation. Here, we demonstrate that Cbl functions as a ubiquitin ligase towards Vav, and that this activity allows Cbl to negatively regulate Vav-mediated signaling.
MATERIALS AND METHODS

Cells

293T human embryonic kidney epithelial cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 20mM HEPES, 1mM sodium pyruvate, 1mM nonessential amino acids, 100units/ml penicillin, 100μg/ml streptomycin, 50μM 2-ME (Life technologies, Gaithersburg, MD) and 10% fetal bovine serum (Hyclone Inc., Logan, UT). JMC-T cell line, an SV40 Large T antigen-expressing derivative of Jurkat-JMC T lymphoma line, was maintained as described (51). Immortal T cell lines 230 and 206, derived respectively from Cbl+/+ and Cbl−/− mice (52), were maintained in RPMI 1640 containing supplements as above for 293T cells.

Antibodies

The antibodies used were: 12CA5, mouse monoclonal (mAb) anti-influenza hemagglutinin (HA) epitope tag (53); 4G10, mAb anti-phosphotyrosine (pY) (54); B-2, mAb anti-Vav (Santa Cruz Biotechnology, Santa Cruz, CA); 4D10, mAb anti-Syk (Santa Cruz Biotech.); M2, mAb anti-FLAG (Invitrogen Corp., Carlsbad, CA); OKT3, mAb anti-CD3 (ATCC); CD28.2, mAb anti-human CD28 (Pharmingen, San Diego, CA); H-211, rabbit polyclonal antibody (pAb) anti-Vav (Santa Cruz Biotech.); C-15, pAb anti-Cbl (Santa Cruz Biotech.); UG9510, pAb anti-ubiquitin (Affiniti Research Products, Exeter, UK).
Expression plasmids

The pAlter-MAX constructs encoding the HA-tagged Cbl proteins, the human Syk-Y323F mutant and the pSRαNeo-CD8ζ chimera (CD8α extracellular and transmembrane domains fused to TCRζ-chain cytoplasmic region) have been described (2). The pAletr-Max HA-Cbl-Y700F and F700Y mutant plasmids (26), the NF-AT firefly luciferase reporter plasmid (55), and the pcDNA3.1-FLAG-Ub plasmid (56) have also been previously described. The pEF-myc-Vav encoding a myc-tagged Vav has been previously described (57), and was used to generate the Vav-Y174F mutant using the Quickchange Mutagenesis system (Invitrogen, Carlsbad, CA).

Transient transfection and cell lysis

293T cells were transfected using the calcium phosphate method, as previously described (52). The cell lysates used for coimmunoprecipitation studies were prepared in Triton lysis buffer (1% Triton X-100 [Fluka, St.Louis, MO], 50mM Tris [pH 7.5], 150mM sodium chloride), whereas those used to assess ubiquitinylation were prepared in the more stringent radioimmunoprecipitation assay (RIPA) buffer (Triton lysis buffer supplemented with 0.1% SDS and 0.5% sodium deoxycholate); each lysis buffer also contained 1mM phenylmethylsulfonyl fluoride, 1mM sodium orthovanadate, 10mM sodium fluoride, and 1µg per ml each of leupeptin, pepstain, antipain, and chymostatin (Sigma, St. Louis, MO). The cell lysate protein concentration was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin as the standard.
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**Immunoprecipitation and immunoblotting**

Immunoprecipitations (IPs) from the indicated amount of cell lysate protein were performed as described (52), using protein A-sepharose 4B beads (Amersham-Pharmacia Biotech, Piscataway, NJ) as an immunosorbent. For immunodepletion studies, cell lysates were incubated with two serial aliquots of antibodies or were mock incubated, together with protein A-sepharose 4B beads, and the resulting immunodepleted or mock-depleted (control) supernatant was used for further IPs. The IPs or cell lysates were resolved on SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes (NEN Life Science Produces, Boston, MA), and serially incubated with the indicated primary antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Cappel-Oreganon Technika, Durham, NC). Enhanced chemiluminescence (ECL) signals were recorded using a light-sensitive film (NEN Life Science Produces). Densitometry was carried out on directly scanned images using ScionImage for Windows™ software (Scion Corporation, Frederick, ML).

**NF-AT luciferase reporter assay**

JMC-T cells were transfected with the NF-AT luciferase reporter together with Cbl and/or Vav expression plasmids using the electroporation method, as previously described (55). Eighteen hours post-transfection, the cells (2 x 10^5 cells/well) were seeded in quadruplicates for each stimulation condition, and stimulated for 6-8 h at 37°C with media alone, 0.25µg/ml anti-CD3 mAb, or 0.25µg/ml each of anti-CD3 plus anti-CD28 mAb. Luciferase assays were performed on equal aliquots of cell lysates according to the manufacturer’s instructions (Promega, Madison, WI). Where indicated,
the cells were cotransfected with a Renilla luciferase reporter plasmid (pRL-TK; Promega). Firefly (NF-AT) and Renilla luciferase activities were measured using a Dual Luciferase assay Kit (Promega), and NF-AT luciferase activity in each sample was normalized to Renilla luciferase activity to control for any variability in the transfection efficiency; the data are presented as mean ± standard deviation of quadruplicate samples. All reporter assays were repeated at least three times. For biochemical analysis, an aliquot of the same cells that were used for the luciferase assay was cultured for a total of 48 h prior to cell lysis.

**Stimulation of mouse T cell lines**

T cell lines 230 and 206 were stimulated by incubating in medium containing 0.2mM sodium orthovanadate or pervanadate (27) at 37°C for the indicated times.
RESULTS

Cbl-dependent Vav ubiquitinylation in T cells

Given the previously described association of Vav with Cbl upon TCR stimulation (27), and the ubiquitin ligase activity of Cbl towards a number of PTKs (14,20,58), we wished to determine if Vav is a target of Cbl-dependent ubiquitinylation. For this purpose, we analyzed a matched pair of virally immortalized T cell lines derived from Cbl$^{+/-}$ (cell line 230) and Cbl$^{-/-}$ (cell line 206) mice, respectively; we and others have previously used these cell lines to demonstrate the Cbl-dependence of Fyn and Lck ubiquitinylation and degradation (17,52), and the inhibitory role of Cbl in the localization of Lck to lipid-rich microdomains (59). As these cell lines do not express the surface TCR (data not shown), we used sodium orthovanadate or sodium pervanadate treatment to induce the activation of these cells. Anti-Vav IPs of unstimulated and pervanadate-stimulated cell lysates were immunoblotted with anti-ubiquitin antibody to detect if Vav was ubiquitinylated. The stimulation of the Cbl$^{+/-}$ cell line (line 230) resulted in the appearance of slower migrating species recognized by the anti-ubiquitin antibody (Fig. 1A, first panel, lanes 3 vs. 4); these signals were substantially enhanced when stimulation was carried out in the presence of the proteasome inhibitor MG132 (Fig. 1B, first panel). In contrast to the Cbl$^{+/-}$ cell line, Vav ubiquitinylation was barely detectable in the pervanadate treated Cbl$^{-/-}$ cell line (line 206) (Fig. 1A, first panel, lane 4 vs. 2); even after treatment in the presence of MG132, the ubiquitin signals were dramatically lower.
Running title: Cbl mediates Vav ubiquitinylation.

compared to those in the Cbl<sup>+/−</sup> cell line (Fig. 1B, first panel, lanes 2 vs. 4). One consequence of ubiquitinylation is to target the substrate proteins for degradation by the 26S proteasome (60,61). In support of such a possibility, the level of ubiquitinylated Vav signal increased when cells were incubated in the presence of MG132 (above). However, stimulation of the Cbl<sup>+/−</sup> or Cbl<sup>−/−</sup> cell lines with sodium orthovanadate did not lead to any detectable decrease in the overall Vav protein levels (Fig. 1C, second panel). Notably, however, orthovanadate stimulation of Cbl<sup>+/+</sup> cell line led to a time-dependent decrease in the level of phosphorylated Vav protein, as shown by anti-pY immunoblotting of anti-Vav IPs; reduction in phospho-Vav levels was visible within 10 min and continued to 30 min (Fig. 1C, first panel, lanes 7-8). In contrast, little if any decrease in phospho-Vav levels was seen in the orthovanadate-treated Cbl<sup>−/−</sup> cell line (Fig. 1C, first panel, lanes3-4). These results strongly suggest that Vav undergoes ubiquitinylation upon cellular activation via PTKs with an accompanying loss of phosphorylated Vav protein, and that Cbl is an important determinant of Vav ubiquitinylation and the concomitant loss of phospho-Vav protein.

The requirement of the Cbl RING finger domain and Y700 for Cbl-dependent Vav ubiquitinylation

The current model of Cbl-dependent ubiquitinylation, based primarily on studies of PTKs, is that Cbl associates with its targets via a protein-protein interaction and juxtaposes the RING finger-associated Ubc (E2) to the target protein. Therefore, we wished to assess if the motifs critical for Cbl/Vav association are required for Vav
ubiquitinylation and if an intact RING finger domain in Cbl is necessary. For this purpose, we established a reconstitution system in 293T cells, utilizing myc-tagged Vav and HA-tagged Cbl. As Vav and Cbl association in T cells is thought to involve the phosphorylation of Y700 in Cbl, we first determined the extent of Cbl/Vav association in transfected 293T cells with or without cotransfection of the relevant PTK, Syk. Syk was selected due to the availability of its mutant, Syk-Y323F, that fails to bind to the Cbl TKB domain and is therefore not subject to Cbl-mediated negative regulation (2,16), which would otherwise complicate the interpretation of the results. In these analyses, CD8ζ was cotransfected as it provides a scaffold for the activation of Syk in non-lymphoid cells (2,16,62). When Cbl and Vav were cotransfected in the absence of Syk, only a low level of association was observed in coimmunoprecipitation analyses (Fig. 2A, top panel, lane1). Concurrent transfection of either wildtype (WT) Syk or the Syk-Y323F mutant led to the expected phosphorylation of Cbl and Vav (second panel, lane 2 and 3), and substantially increased the level of Cbl/Vav association (top panel, lanes 2 and 3). Thus, we chose the Syk-Y323F mutant for further analyses of Cbl/Vav interactions.

To assess if Cbl can target Vav for ubiquitinylation in the 293T cell reconstitution system, we transfected 293T cells with myc-Vav with or without HA-Cbl and Syk-Y323F, in addition to a FLAG-tagged ubiquitin (FLAG-Ub) plasmid. Anti-FLAG immunoblotting of anti-Vav IPs was used to detect the ubiquitinylated Vav, which appears as higher molecular weight species reactive with an anti-FLAG antibody. Relatively low ubiquitinylated Vav signals were observed in the absence of cotransfected Syk-Y323F either with or without Cbl (Fig. 2B, top panel, lanes 1 and 2). Cotransfection
of Syk-Y323F without Cbl resulted in a slight enhancement of the Vav ubiquitin signal (lane 3); however, a dramatic increase in the ubiquitinated Vav signal was seen when both Cbl and Syk-Y323F were cotransfected (lane 4). As observed in the Cbl\(^{+/+}\) T cell line above (Fig. 1A, bottom panel), the enhanced Vav ubiquitination was not accompanied by a detectable decrease in Vav protein levels (Fig. 2B, second panel). However, when the same membrane was reprobed with an anti-pY antibody, a reduced phospho-Vav signal was detected in cells cotransfected with Cbl (third panel, compare lane 3 vs. 4), again suggesting that ubiquitination may specifically target the active (phosphorylated) pool of Vav for degradation. Consistent with this possibility, treatment of transfected 293T cells with the proteasome inhibitor MG132 partially countered the Cbl-dependent reduction in phospho-Vav levels (Fig. 2C, third panel, compare lanes 2 and 4); concomitantly, MG132 treatment resulted in an increase in the level of ubiquitinylated Vav signals (top panel, compare lanes 1 vs. 3 and 2 vs. 4).

To confirm that the slower migrating ubiquitinylated species represented Vav, and not cotransfected Cbl or Syk, we first resorted to immunoblotting with the respective antibodies. While Cbl and Syk signals were not detectable, the slower migrating species could not be categorically identified as Vav, apparently reflecting the small pool of Vav that is ubiquitinylated (data not shown). We therefore used an immunodepletion strategy to confirm that the observed ubiquitinylated species represent Vav and not associated Cbl or Syk proteins. Two rounds of immunodepletion resulted in greatly reduced Cbl levels and essentially undetectable levels of Syk in the cell lysates, compared to the mock-depleted lysates (Fig. 2D, bottom two panels). However, such immunodepletion had no
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effect on the levels of ubiquitinylated species detected in anti-Vav IPs, strongly suggesting that these represent ubiquitinylated Vav, and not the ubiquitinylated Cbl or Syk proteins. Overall, the results presented above indicate that Cbl, in the presence of a relevant tyrosine kinase, can target Vav for ubiquitinylation and subsequent degradation of the ubiquitinylated Vav pool.

Previous studies by Penninger and colleagues showed that the Cbl/Vav association in T cells was activation-dependent, and in vitro competition with phosphopeptides suggested that the interaction was mediated by the Vav SH2 domain binding to a conserved pY^{700}MTP motif in Cbl (27). However, this conclusion has not been formally demonstrated in vivo. Thus, we first utilized the 293T cell cotransfection system to conclusively define the mode of Cbl/Vav association. Anti-HA immunoblotting of anti-Vav IPs from lysates of 293T cells cotransfected with HA-Cbl and Vav showed that intact TKB (using the Cbl-G306E mutant) and RING finger (using the Cbl-C3AHN mutant) domains were not required for the Cbl/Vav association (Fig. 3A, top panel, lanes 3 and 4 vs. lane 2). Importantly, a truncated Cbl mutant with intact TKB and RING finger domains as well as the proline-rich region (Cbl-655) did not associate with Vav, indicating that the Vav-binding site(s) resided within the C-terminal region of Cbl where the major phosphorylation sites are located. Indeed, a Cbl mutant (Cbl-Y5F) (26) with mutations of three well established (Y700, Y731 and Y774) and two putative (Y674 and Y735) phosphorylation sites was unable to associate with Vav (lane 5).

Consistent with previous in vitro studies implicating Cbl Y700 as a potential binding site for the Vav SH2 domain, we found that the Cbl-Y700F mutant was unable to associate
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with Vav (lane 6). Remarkably, the ability of the Cbl-Y5F mutant to associate with Vav was restored by adding back Y700 (Fig. 3C, top panel, lane 3 vs. 2). As a control, we demonstrated that HA-Cbl was not coimmunoprecipitated with an irrelevant antibody (Figure 3B), and that comparable levels of various HA-Cbl proteins were expressed in transfected cells as determined by anti-HA immunoblotting of cell lysates (Figure 3 A-C, bottom panels). These results conclusively demonstrate that the Cbl/Vav association is mediated by Vav binding to phosphorylated Y700 on Cbl.

Using the ubiquitylation assay in 293T cells (as described above), we found that the Y700F mutation markedly reduced the ability of Cbl to mediate Vav ubiquitylation (Fig. 3D, lane 5 vs. 3). Importantly, an intact RING finger domain was required for Cbl to induce Vav ubiquitylation (lane 4). Thus, Cbl-mediated ubiquitylation of Vav is critically dependent on the structural motifs within Cbl that mediate its physical interactions with the target (Vav) and the E2 enzyme of the ubiquitin pathway.

Enhanced Cbl-dependent ubiquitylation of the activated Vav mutant, Vav-Y174F

One obvious reason for the requirement of a cotransfected PTK (Syk-Y323F) for Vav ubiquitylation (above) is to induce Cbl phosphorylation, which in turn mediates the Cbl/Vav association (27). However, the Cbl-dependent decrease in phospho-Vav levels noted above (Fig. 1C and 2B) and increased phospho-Vav levels in both Cbl-b and Cbl/Cbl-b deficient T cells (23,25,50), suggest that phosphorylation-dependent activation may render Vav more susceptible to Cbl-dependent ubiquitylation. Y174 in Vav
mediates a crucial intramolecular interaction that inhibits Vav activity, and its phosphorylation enhances the Vav activity by promoting an open conformation (47). Notably, the Y174F mutation induces the open conformation of Vav, and renders it constitutively active and oncogenic (48,49). We therefore used the Vav-Y174F mutant to assess if the activated conformation of Vav protein is more susceptible to Cbl-dependent ubiquitinylation. Notably, the level of association of Cbl with the Vav-Y174F mutant was substantially higher compared to WT Vav when analyzed in the absence of cotransfected Syk-Y323F (Fig. 4A, lane 1 vs. 3); comparable levels of association with Cbl were observed when Syk-Y323F was cotransfected (Figure 4A, compare lane 2 and 4). Importantly, the level of Cbl-dependent ubiquitinylation of Vav-Y174F mutant was substantially higher compared to that of WT Vav, both in the absence (Figure 4B, top panel, compare lane 1 and 4) and in the presence of cotransfected Syk-Y323F (compare lane 3 and 6). These results further support the idea that Cbl may preferentially bind to the activated form of Vav and target it for ubiquitinylation.

The fact that Vav-Y174F mutant associated with Cbl and was more susceptible to Cbl-dependent ubiquitinylation strongly suggested that phosphorylation of Y174 itself was not responsible for Cbl/Vav association or subsequent Vav ubiquitinylation. To directly ascertain if the Vav-Y174F mutant associates with Cbl via a mechanism similar to that of WT Vav, we compared WT Vav with Vav-Y174F mutant for association with WT vs. Y700F mutant Cbl. As observed in Fig. 3A and C, Cbl-Y700F mutant association with WT Vav was greatly reduced (Fig. 5A, top panel, lane 3). Importantly, the level of association of Vav-Y174F with Cbl was also markedly reduced by the Y->F
mutant of Y700 (Fig. 5A, top panel, compare lane 5 and 6). Interestingly, the residual level of association between Vav-Y174F and Cbl-Y700F was relatively higher compared to the association of WT Vav with Cbl-Y700F (Fig. 5A, top panel, compare lane 3 and 6). It is possible that the open conformation of Vav promotes additional mechanisms of Cbl/Vav association distinct from that mediated via Cbl Y700. In agreement with the association results, the ability of Cbl-Y700F mutant to induce the ubiquitinylation of Vav-Y174F was markedly reduced compared to that of WT Cbl (Fig. 5B, top panel, compare lane 5 and 6). In keeping with a higher level of residual association between Cbl-Y700F and Vav-Y174F mutants, Cbl-Y700F mutant induced a higher level of ubiquitinylation of Vav-Y174F mutant than that of WT Vav (Fig. 5B, top panel, compare lane 3 and 6). The results further support the idea that Cbl bind to the open conformation of Vav and induces its ubiquitinylation.

Cbl inhibits Vav-dependent NF-AT activation

Given our findings that Vav undergoes PTK activation and Cbl-dependent ubiquitinylation and that phospho-Vav is degraded under these conditions, we wished to ask if Cbl indeed might function as a negative regulator of Vav signaling. Among the various functional readouts of Vav-dependent signaling, the transcriptional activation of the IL-2 promoter provides a convenient and sensitive assay. To this end, we utilized a reporter incorporating the NF-AT-binding sites of the IL-2 promoter linked to the firefly luciferase gene. Overexpression of Vav is known to increase the level of TCR-induced NF-AT-luciferase activity (31), allowing an analysis of the effect of Cbl in the contexts
of Vav function. Thus, we carried out transient transfection of Jurkat T cells with plasmids encoding Vav and various Cbl proteins, together with the NF-AT-luciferase reporter. Overexpression of Vav led to a substantial increase of basal as well as the anti-CD3 or anti-CD3 plus anti-CD28 induced NF-AT-luciferase activity, compared with that in the vector-transfected control cells (Fig. 6A). Cotransfection of wild type Cbl substantially reduced the Vav-induced increase in basal as well as anti-CD3 or anti-CD3 plus anti-CD28 induced NF-AT-luciferase activity. Importantly, the Cbl-C3AHN mutant, lacking the ubiquitin ligase activity (16,62), failed to inhibit the Vav-dependent NF-AT-luciferase activity. The expression levels of Vav and Cbl proteins were confirmed by immunoblotting (Fig. 6B). These results support the role of Cbl as a negative regulator of Vav function through a mechanism that requires the ubiquitin ligase activity of Cbl.
DISCUSSION

Members of the Vav family (Vav, Vav2 and Vav3) of Rac/Rho GEFs have emerged as crucial signaling elements of tyrosine kinase-coupled cell surface receptors. The hematopoietic-restricted Vav protein is crucial for lympho-hematopoietic development and activation, and mutant forms of Vav can mediate oncogenesis. Thus, a better understanding of the biochemical mechanisms of Vav regulation is of considerable biological interest. Prior studies have shown that TCR stimulation induces the association of Vav with Cbl, which has recently emerged as a negative regulator of receptor and nonreceptor tyrosine kinases by virtue of its ubiquitin ligase activity (12,14,16-18,20). Here, we have elucidated the mechanism by which Cbl interacts with Vav. We demonstrated that Cbl/Vav association leads to the ubiquitinylation and degradation of active Vav, and show that Cbl can function as a negative regulator of Vav-mediated cellular activation.

We used immortal Cbl^{+/+} and Cbl^{-/-} T cell lines to establish that Vav undergoes Cbl-dependent ubiquitinylation and degradation when cellular tyrosine kinase pathways are activated. Complementary analyses in transfected 293T cells confirmed the ability of Cbl to target Vav for ubiquitinylation. Furthermore, analyses in both systems demonstrated that Cbl-dependent ubiquitinylation of Vav required tyrosine phosphorylation (by orthovanadate or pervanadate treatment, or cotransfection of Syk-Y323F). One likely explanation for the requirement for tyrosine phosphorylation was the postulated mechanism of Cbl/Vav interaction, namely via the Vav SH2 domain binding
Running title: Cbl mediates Vav ubiquitinylation.

to pY700 of Cbl (27). We used a panel of Cbl mutants to directly demonstrate that phosphorylation of Cbl Y700 was crucial as well as the predominant mechanism for Cbl/Vav association (Fig. 3A, C). Importantly, abrogation of the Cbl/Vav association via Cbl pY700 mostly eliminated the Cbl-dependent Vav ubiquitinylation. The low level of residual Vav ubiquitinylation in the presence of the Y700F Cbl mutant (Fig. 3D) may represent the existence of minor additional mechanisms of association, such as either the Vav SH3 domain binding to the Cbl proline-rich region or the Vav SH2 domain binding to other phosphorylation sites on Cbl. Thus, the phosphorylation dependence of the Cbl/Vav association provides one likely mechanism for the requirement of tyrosine kinase activation for Cbl-dependent Vav ubiquitinylation. Furthermore, our analyses of an activated mutant of Vav (Vav-Y174F) showed it to be more susceptible to Cbl-dependent ubiquitinylation. This mutant was fully capable of associating with Cbl, indicating that phosphorylated Y174 itself does not mediate Cbl/Vav association. Cbl-Y700F mutation severely impaired the association of Vav-Y174F mutant with Cbl and its subsequent ubiquitinylation; thus the active, open conformation of Vav associates with Cbl via phosphorylated Cbl-Y700. A key mechanism that regulates the physiological activation of Vav is phosphorylation of regulatory tyrosine residues, including Y174, which leads to removal of the autoinhibition induced by the N-terminal domain interacting with the DH domain. Thus, the more open structure of Vav may either promote a more effective Cbl/Vav association (see Fig. 4A and 5A) or expose target lysine residues on Vav for Cbl-dependent ubiquitinylation. Regardless of their relative contributions, these mechanisms would ensure that Cbl selectively targets the activated pool of Vav for ubiquitinylation, a theme reminiscent of Cbl-dependent ubiquitinylation.
of tyrosine kinases (12,16,17,58,63). A further parallel is provided by the requirement of an intact Cbl RING finger domain for Cbl-dependent ubiquitinylation of Vav. These findings favor a model of Cbl-dependent Vav regulation analogous to that of Cbl-dependent regulation of tyrosine kinases: an activation-dependent association step juxtaposes Cbl with its target (Vav) leading to its ubiquitinylation followed by functional regulation mediated by the ubiquitin tag.

In both the Cbl\(^{+/+}\) and the Cbl\(^{-/-}\) T cell lines, as well as the 293T transfection system, proteasome inhibitor treatment led to the accumulation of ubiquitinylated Vav, indicating that Cbl-dependent ubiquitinylation targets Vav for proteasomal degradation. However, an overall decrease in the levels of Vav protein was not seen under conditions where Vav ubiquitinylation was clearly observed. Given that the Cbl/Vav association and ubiquitinylation required tyrosine kinase activation, and that Vav activity is regulated by tyrosine phosphorylation, we reasoned that only the activated pool may be subjected to Cbl-dependent ubiquitinylation and degradation. Consistent with this idea, Cbl-dependent reduction of phosphorylated Vav level was observed in the Cbl\(^{+/+}\) but not the Cbl\(^{-/-}\) T cell line, and in Cbl-transfected 293T cells. Thus, we favor the idea that Cbl-dependent ubiquitinylation of Vav targets its activated pool for degradation. This idea is consistent with the current models of Vav activation by tyrosine phosphorylation. However, it remains possible that non-phosphorylated Vav may manifest biological activity under certain conditions; given the phosphorylation-dependent nature of Cbl/Vav association, the putative biological activities of non-phosphorylated Vav are unlikely to be regulated by Cbl.
Cbl-b was shown to induce the ubiquitinylation of another non-tyrosine kinase target, PI 3-kinase, which also associates with Cbl proteins via a phosphorylation site. However, the authors did not observe Cbl-dependent changes in the levels of PI 3-kinase subunits or an effect of proteasome inhibitors, leading to the suggestion that Cbl-b inhibits the PI 3-kinase activity independent of degradation, although specific degradation of a small pool of activated PI 3-kinase could have gone undetected. Alternatively, Cbl-dependent ubiquitinylation of different target proteins may indeed lead to different biological consequences, as further exemplified by the Cbl-dependent ubiquitinylation of receptor tyrosine kinases, which provides a tag for lysosomal targeting (10,12-14).

It has been previously established that Vav participates in the induction of NF-AT/AP1-mediated transcription, and this function requires its GEF activity (64,65). We therefore used an NF-AT-luciferase reporter assay in Jurkat T cells to demonstrate that Cbl functions as a negative regulator of Vav-dependent cellular activation. Importantly, Cbl-mediated inhibition of Vav-induced NFAT-luciferase reporter activity required an intact RING finger domain, strongly supporting the role of Cbl-mediated ubiquitinylation in functional regulation of Vav. Consistent with the role of Cbl as a negative regulator of Vav, recent studies have shown that unstimulated as well as anti-CD3 stimulated thymocytes from Cbl−/− mice have markedly higher levels of Rac-GTP compared to those in Cbl+/+ thymocytes (66). Notably, activated Cbl-b−/− T cells also showed higher levels of phospho-Vav and increased TCR clustering, a phenomenon dependent on Rac
Running title: Cbl mediates Vav ubiquitinylation.

activation (23,50). As PI-3 kinase products are important in promoting the activation of Vav, loss of PI 3-kinase function could indirectly reduce the overall level of activated Vav. Our studies, however, establish a direct mechanism for Cbl-induced negative regulation of Vav. It is likely that in a physiological context, both direct and indirect mechanisms cooperate to mediate a more effective and integrated regulatory effect. Given that two Cbl family members can potentially regulate Vav and that three Vav family members exist, further studies will be necessary to precisely elucidate the redundant versus specific functional roles of the interactions that take place between the Cbl and Vav families of proteins. While Cbl<sup>-/-</sup> mice have significant abnormalities in T cell development, Cbl-b<sup>-/-</sup> mice show normal T cell development but a profound hypersensitivity of peripheral T cells to TCR triggering (23). It is notable that Cbl mRNA expression in thymocytes is far greater than that of Cbl-b, whereas this is not the case in peripheral T lymphocytes (8,25). Recent studies have demonstrated that two other proteins, SOCS1 and hSIAH2, can also negatively regulate Vav via ubiquitinylation (57,67). Whether Cbl functions independently of or together with these other ubiquitin ligases are important questions for future studies.

In conclusion, we present evidence that Cbl functions as a negative regulator of Vav by targeting activated Vav for ubiquitinylation and degradation.
Acknowledgements

This work was supported by the NIH grants to HB (CA 87986, CA 76118, CA 99900, CA99163 and CA75075) and VB (CA 81076 and CA 70195), and the US Army Breast Cancer Research Program trainee award DAMD17-99-1-9086 (LD).

We thank Dr. Yoshihide Kanaoka, Dr. Ingrid Dodge and Dr. Maria Simarro for discussion, and Dr. Ingrid Dodge for reading the manuscript. We thank other members of the Band laboratory for helpful suggestions.
Running title: Cbl mediates Vav ubiquitylation.

**Abbreviations:**

PTK, protein tyrosine kinase; IP, immunoprecipitate; IB, immunoblot; mAb, monoclonal antibody; RTK, receptor tyrosine kinases; WT, wildtype; TKB, tyrosine kinase-binding; SH, Src-homology; PH, plekstrin-homology; pY, phosphotyrosine; TCR, T cell antigen receptor.
REFERENCES


Running title: Cbl mediates Vav ubiquitinylation.


Abbreviations:
FIGURE LEGENDS

Fig. 1. Pervanadate stimulation induces Vav ubiquitinylation and loss of phospho-Vav in Cbl+/+ but not in Cbl−/− T cells. A. Immortal Cbl−/− (206) and Cbl+/+ (230) T cell lines were either left untreated (0 min) or treated with 0.2mM sodium pervanadate for 20 min at 37°C, and then lysed in PIRA buffer. Anti-Vav immunoprecipitations (IPs) from 0.5mg aliquots of lysate proteins were immunoblotted with anti-ubiquitin (Ub) antibody (top panel), followed by anti-Vav antibody (second panel). NS, nonspecific. B. The Cbl−/− and Cbl+/+ cell lines were treated with DMSO control (-) or 50μM MG132 (MG132) for 3 h and then treated with 0.2mM sodium pervanadate for 20 min at 37°C prior to cell lysis in RIPA buffer. Anti-Vav IPs from 0.5mg aliquots of lysate proteins were immunoblotted with anti-Ub antibody (first panel), followed by anti-Vav antibody (second panel). C. The Cbl−/− and Cbl+/+ cell lines were treated with 0.2mM sodium vanadate for the indicated times. Anti-Vav IPs from 200μg aliquots of RIPA lysate proteins were immunoblotted with anti-pY antibody (top panel), followed by anti-Vav antibody (bottom panel). The level of phosphorylated Vav (top panel) and total Vav protein (second panel) were quantified by densitometry and the values are presented as fraction of the values for 0 min treatment (value of 1).

Fig. 2. Syk-dependent Vav ubiquitinlylation by Cbl in 293T cells. A. 293T cells were transfected with 0.5μg of Vav, 2μg of HA-Cbl and 0.5μg of CD8ζ expression plasmids with or without 50ng of WT-Syk or Syk-Y323F plasmids. 48 h post-transfection, cells were washed once with phosphate-buffered saline and lysed in Triton lysis buffer. Anti-
Vav IPs from 1mg aliquots of lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-HA antibody (top panel), followed by anti-pY antibody (second panel) and anti-Vav antibody (third panel). 25µg aliquots of cell lysates were similarly resolved and immunoblotted with anti-Syk antibody (fourth panel) and anti-HA antibody (bottom panel). B. 293T cells were transfected as in A, using Vav, CD8ζ and 5µg of FLAG-Ub, with (+) or without (-) HA-Cbl and/or Syk-Y323F, as indicated. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody (top panel), followed by anti-Vav (second panel) and anti-pY (third panel) antibodies. 25µg aliquots of cell lysates were similarly resolved and immunoblotted with anti-HA antibody. HC, heavy chain. C. 293T cells were transfected as in A, using Vav, CD8ζ, Syk-Y323F and FLAG-Ub with (+) or without (-) HA-Cbl. Cells were treated with 50µM MG132 (+) or DMSO control (-) for 4h prior to harvesting the cells. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG antibody (first panel), followed by anti-Vav (second panel) and anti-pY (third panel) antibodies. HC, heavy chain. D. 293T cells were transfected as in A, using Vav, CD8ζ and FLAG-Ub, with (+) or without (-) Syk-Y323F and/or HA-Cbl. The RIPA lysates were subjected to two serial anti-Cbl or anti-Syk IPs (immunodepletion), and then 1mg aliquots of the mock-depleted or immunodepleted lysates were subjected to anti-Vav IPs, resolved by SDS-PAGE and immunoblotted with anti-Flag (first panel), followed by anti-Vav (second panel) antibodies. 25µg aliquots of mock-depleted or immunodepleted cell lysates were directly immunoblotted with anti-Cbl (third panel) and anti-Syk (fourth panel) antibody.
Fig. 3. **The requirements for the Cbl/Vav association through Cbl Y700, and Cbl RING finger domain for ubiquitinylation of Vav.**  

**A.** 293T cells were transfected with 0.5 µg of Vav, 2 µg of wild type (WT) or mutant HA-Cbl construct and 0.5 µg of CD8ζ, with or without 50 ng of Syk-Y323F. 48 h post-transfection, cells were washed once with phosphate-buffered saline and lysed in Triton lysis buffer. Anti-Vav IPs from 1 mg aliquots of lysate proteins were resolved by SDS-PAGE and immunoblotted with anti-HA (top panel) and anti-Vav (second panel) antibodies. 25 µg aliquots of cell lysates were immunoblotted with anti-HA antibody (lower panel).

**B.** 293T cells were transfected as in A, using HA-Cbl, CD8ζ, Vav and Syk-Y323F. Anti-Vav or isotype-matched control antibody IPs from 1 mg aliquots of Triton lysate proteins were immunoblotted with anti-HA antibody (top panel), followed by anti-Vav antibody (second panel). 25 µg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel).

**C.** 293T cells were transfected as in A, using WT or mutant HA-Cbl construct, Vav, CD8ζ and Syk-Y323F. Anti-Vav IPs from 1 mg aliquots of Triton lysate proteins were immunoblotted with anti-HA (first panel) and anti-Vav (second panel) antibodies. 25 µg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel).

**D.** 293T cells were transfected as in A, using Vav, CD8ζ, Syk-Y323F and 5 µg of FLAG-Ub, with or without various HA-Cbl construct. Anti-Vav IPs from 1 mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG (first panel) and anti-Vav (second panel) antibodies. 25 µg aliquots of cell lysates were immunoblotted with anti-HA antibody (lower panel). HC, heavy chain.
Fig. 4. Enhanced Cbl-dependent ubiquitinylation of the activated Vav mutant, Vav-Y174F. A. 293T cells were transfected with 0.5μg of WT Vav or Vav-Y174F and 2μg of HA-Cbl, 0.5μg of CD8ζ, with (+) or without (-) 50ng of Syk-Y323F, and cell lysates were prepared 48h post-transfection. Anti-Vav IPs from 1mg aliquots of Triton lysate proteins were immunoblotted with anti-HA (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysate proteins were directly immunoblotted with anti-HA antibody (lower panel). B. 293T cells were transfected as in A, using WT Vav or Vav-Y174F, CD8ζ and 5μg of FLAG-Ub, with (+) or without (-) HA-Cbl and/or Syk-Y323F. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel). HC, heavy chain.

Fig. 5. The requirement for the Cbl Y700-mediated Cbl/Vav association in Cbl-dependent enhanced ubiquitinylation of Vav-Y174F mutant. A. 293T cells were transfected with 0.5μg of WT Vav or Vav-Y174F, 0.5μg of CD8ζ, 50ng of Syk-Y323F and 2μg of WT Cbl or Cbl-Y700F, and cell lysates were prepared 48h post-transfection. Anti-Vav IPs from 1mg aliquots of Triton lysate proteins were immunoblotted with anti-HA (top panel) and anti-Vav (second panel) antibodies. 25μg aliquots of cell lysate proteins were directly immunoblotted with anti-HA antibody (lower panel). B. 293T cells were transfected as in A, using WT Vav or Vav-Y174F, CD8ζ, Syk-Y323F and 5μg of FLAG-Ub, and WT Cbl or Cbl-Y700F. Anti-Vav IPs from 1mg aliquots of RIPA lysate proteins were immunoblotted with anti-FLAG (top panel) and anti-Vav (second
panel) antibodies. 25μg aliquots of cell lysates were directly immunoblotted with anti-HA antibody (lower panel).

Fig. 6. **Inhibition of Vav-dependent transactivation of NF-AT-luciferase reporter by Cbl, and the requirement of the Cbl RING finger domain.** A. Jurkat-derived JMC-T cells were transfected with plasmids encoding the NF-AT-luciferase reporter (10μg), Vav (10μg) and either WT or the RING finger mutant (C3AHN) of HA-Cbl (10μg), together with pRL-TK plasmid (10ng) encoding Renilla luciferase as a transfection efficiency control. 18 h post-transfection, cells were plated in replicates of four and either left unstimulated or stimulated with anti-CD3 antibody with or without anti-CD28 antibody. NF-AT (*firefly*) luciferase activity in each sample was normalized to *Renilla* luciferase activity, and the data are presented as mean ± standard deviation of quadruplicates. All reporter assays were repeated at least three times, with similar results. B. Aliquots of the cell lysates used for luciferase assay were harvested after 48 h and 100μg aliquots of lysate proteins were used for immunoblotted with anti-HA and anti-Vav antibody.
Fig 1

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anti-Vav IP
anti-Ub blot

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anti-Vav Blot

Relative Vav Protein level

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Fig. 3

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anti-Vav IP
anti-HA blot
83

anti-Vav IP
anti-Vav blot
lysate
anti-HA blot
83

Lane: 1 2 3 4 5 6 7

B

IP:
control
anti-Vav

anti-HA blot

anti-Vav blot

lysate
anti-HA blot

Lane: 1 2

C

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anti-Vav IP
anti-Vav blot
lysate
anti-HA blot

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anti-FLAG blot
175

anti-Vav IP
anti-Vav blot
lysates
anti-HA blot

Lane: 1 2 3 4 5

Vav-Ub

HC
Fig. 4

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| anti-Vav IP |
| anti-Vav blot |
| lysates |

Lane: 1 2 3 4 5 6
Fig. 5

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- lysates
- anti-HA blot

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- anti-Vav IP
- anti-Vav blot
- lysates
- anti-HA blot

Lane: 1 2 3 4 5 6

Vav-Ub

175
83
Fig. 6

A

NFAT-Luc activity, normalized RLU

Vav: - + + +
HA-Cbl: - - WT C3AHN

B

Vav: - + + +
HA-Cbl: - - WT C3AHN

lysates
anti-Vav blot

lysates
anti-HA blot

Lane: 1 2 3 4
Cbl-mediated ubiquitinylation and negative regulation of Vav
Yuko Miura-Shimura, Lei Duan, Navin L. Rao, Alagarsamy Lakku Reddi, Hideki Shimura, Rob Rottapel, Brain J. Druker, Alexander Tsygankov, Vimla Band and Hamid Band

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