Cbl Promotes Ubiquitination of the T Cell Receptor ζ through an Adaptor Function of Zap-70*

Hong-Ying Wang, Yoav Altman, Deyu Fang, Chris Elly, Yang Dai, Yuan Shao, and Yun-Cai Liu‡

From the Division of Cell Biology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

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Triggering of the T cell antigen receptor (TCR)–CD3 complex induces its ubiquitination. However, the molecular events that lead to ubiquitin conjugation to these cell surface molecules have not been defined. Here we report that Cbl, a RING-type E3 ubiquitin-protein ligase, promotes ubiquitination of TCRζ chain, which requires its functional variant Src homology 2 domain and an intact RING finger. The tyrosine kinase Zap-70, which binds to both TCRζ and Cbl, plays an adaptor role in these events. Mutations in TCRζ, Zap-70, or Cbl that disrupt the interaction between TCRζ and Zap-70 or between Zap-70 and Cbl reduce ubiquitination of TCRζ. Our results suggest a novel mechanism by which Cbl negatively regulates T cell development and activation by inducing ubiquitination of the TCR-CD3 components.

T cell activation is initiated by the binding of antigenic peptides presented by major histocompatibility complexes (MHCs)1 on the antigen presenting cells to the T cell receptor (TCR) (1, 2). The TCR is assembled as a multiple subunit complex that consists of the specific antigen-binding TCR α and β heterodimer, signal-transducing invariant CD3γ, δ, ε chains, and the structurally distinct TCRζ chain. The signal capacity of the CD3 subunits and TCRζ chain is achieved through the phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs), characterized by the presence of a pair of immunoreceptor tyrosine-based activation motifs (ITAMs), characterized by the presence of a pair of phosphotyrosine residues on the ITAMs through the src homology 2 domain; HA, hemagglutinin.

Ubiquitination of cell surface receptors represents a general mechanism of turning off signal transduction induced by the ligand binding (15). Particularly, a family of receptor tyrosine kinases such as the receptors for epidermal growth factor, platelet-derived growth factor, and colony-stimulating factor-1 go through ubiquitination upon receptor engagement, and this Ub conjugation process involves Cbl, an adaptor protein (16–18). Cbl, a 120-kDa proto-oncogene product, was originally isolated as a cellular homologue of v-Cbl, a part of the transforming fusion protein of CAS NS-1 retrovirus causing leukemia in mice (19). Cbl consists of an N-terminal variant SH2 domain, a RING finger, and a C-terminal proline-rich domain with potential tyrosine phosphorylation sites. Indeed, genetic and biochemical studies have shown that Cbl family proteins including those from Drosophila and Caenorhabditis elegans attenuate intracellular signaling induced by the engagement of cell surface receptors (20). It is now understood that Cbl functions as an E3 Ub ligase with a RING finger that recruits a Ub-conjugating enzyme or E2 and an SH2 domain that recognizes activated receptor tyrosine kinases for Ub conjugation (18, 21–23).

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† To whom correspondence should be addressed: Division of Cell Biology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. E-mail: yuncai@liai.org.
‡ The abbreviations used are: MHC, major histocompatibility complex; TCR, T cell receptor; ITAM, immunoreceptor tyrosine-based activation motif; PTK, protein tyrosine kinase; Ub, ubiquitin; E2, Ub-conjugating enzyme; E3, Ub-protein ligase; SH2 domain, Src homology 2 domain; HA, hemagglutinin.

1 The abbreviations used are: MHC, major histocompatibility complex; TCR, T cell receptor; ITAM, immunoreceptor tyrosine-based activation motif; PTK, protein tyrosine kinase; Ub, ubiquitin; E2, Ub-conjugating enzyme; E3, Ub-protein ligase; SH2 domain, Src homology 2 domain; HA, hemagglutinin.
Two genetic studies using Cbl gene-targeted mice showed that the cell surface expression of the TCR-CD3 complex is up-regulated in Cbl-deficient thymocytes (24, 25). Cbl deficiency favors positive selection of thymocytes (25), suggesting an important role for Cbl in T cell development and activation. The fact that TCRζ chain is ubiquitinated and that Cbl acts as a RING-type E3 ligase for receptor tyrosine kinases prompted us to investigate whether Cbl also functions as an E3 for TCRζ chain to promote its ubiquitination. Here we show that Cbl indeed induces Ub conjugation to TCRζ chain, which requires a functional RING finger and the N-terminal variant SH2 domain of Cbl. Furthermore, mutations of TCRζ ITAMs reduce its ubiquitination, whereas coexpression of Zap-70 enhances the event. Significantly, it is shown that Zap-70 plays an adaptor role in Cbl-induced Ub conjugation to TCRζ chain. Our results provide a molecular mechanism by which Cbl regulates T cell function by promoting ubiquitination of TCRζ chain.

**EXPERIMENTAL PROCEDURES**

Antibodies—Polyclonal anti-Cbl, anti-TCRζ chain, anti-Zap-70 antibodies, anti-Myc, and anti-HA monoclonal antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-TCRζ chain monoclonal antibody was from Transduction Laboratories (Lexington, KY). Anti-Xpress antibody was from Invitrogen (Carlsbad, CA). Anti-human CD3ε (OKT3) monoclonal antibody was purified from ascites using a protein G-Sepharose column. Anti-TCRζ monoclonal antibody (H146-968) was provided by R. Kubo.

Plasmids—The human TCRζ chain cDNA was amplified by polymerase chain reaction using a human lymphocyte cDNA library (CLONTECH) as the template and was subcloned into a pEF vector with an in-frame Myc epitope tag at the 3′-end. A truncated mutant with the second half of the second ITAM and the third ITAM removed (TCRζ 1–121) was amplified by polymerase chain reaction and subcloned into the pEF vector. Point mutations at the N-terminal tyrosine residue to phenylalanine at each of the three ITAMs in wild-type TCRζ (AYF, ABYF, ARCYF) or TCRζ 1–121 (AYF), or lysines 115 and 117 mutated to arginine in TCRζ 1–121 (TCRζ 1–121KR), were generated by site-directed mutagenesis (QuickChange, Stratagene). The human Zap-70 cDNA was subcloned into pEF without or with an Xpress epitope. Point mutations at tyrosine 292 to phenylalanine in Zap-70 were made by site-directed mutagenesis. The HA epitope-tagged Ub cDNA has been described (26, 27). The Cbl cDNAs encoding the full-length Cbl or Cbl G306E without or with an HA epitope in the pEF vector were described previously (28). A Cbl construct containing the N-terminal variant SH2 domain and the RING finger (SH2+RING, amino acids 1–481), or the RING finger and C-terminal proline-rich domain (RING+C, amino acids 355–906) was amplified by polymerase chain reaction and subcloned into pEFNeo with an HA epitope at the 5′-end. Mutations at glutamic acid 306 to alanine (E306A), cysteine 381 to alanine (C381A), and tryptophan 408 to alanine (W408A) in the full-length Cbl or in Cbl 1–481 were generated by site-directed mutagenesis.

**Cell Culture, Transfection, and Stimulation**—Jurkat T cells or a Zap-70-deficient Jurkat T cell line (P116; kindly provided by Dr. Bob Abraham, Duke University, Durham, NC) were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics. For protein expression in Jurkat T cells, cells were transfected with the appropriate amount of plasmids (usually 1–10 μg) by electroporation (240 V, 960 microfarads; Bio-Rad). 293T kidney embryonic cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. For protein expression in 293T cells, cells were transfected with the appropriate amount of plasmids (usually 1–4 μg) by calcium precipitation. After 48 h, cells were collected, resuspended (2 × 10^6/ml) in 0.5 ml of medium, and treated with pervanadate, OKT3, or MG132 (50 μM) for 30 min at 37 °C. Cells were then pelleted and resuspended in 1× Nonidet P-40 lysis buffer (1% Nonidet P-40, 20 mM Tris·HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM Na3P, 2 mM NaVO4, and 10 μg/ml each aprotinin and leupeptin). Cells were lysed for 10 min at 4 °C, and insoluble materials were removed by centrifugation at 15,000 × g (4 °C for 10 min). For displaying ubiquitinating protein bands, 0.1% SDS was added into lysis buffer to disrupt nonspecific protein-protein interaction.

**Immunoprecipitation and Immunoblotting**—For immunoprecipitation, lysates (~1 × 10^7 cells) were mixed with antibodies (1 μg) for 2 h followed by the addition of 30 μl of protein G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h at 4 °C. Immunoprecipitates were washed four times with 1× Nonidet P-40 lysis buffer and boiled in 20 μl of 2× Laemmli’s buffer. Samples were subjected to 8 or 10% SDS-polyacrylamide gel electrophoresis analysis, and electrotetransferred onto polyvinylidene difluoride membranes (Millipore). Membranes were probed with the indicated primary antibodies (usually 1 μg/ml), followed by horseradish peroxidase-conjugated secondary antibodies. Membranes were then washed and visualized with enhanced chemiluminescence detection system (ECL, Amersham Pharmacia Biotech). When necessary, membranes were stripped by incubation in stripping buffer (62.5 mM Tris·HCl, pH 5.7, 100 mM 2-mercaptoethanol, 2% SDS) for 1 h at 70 °C with constant agitation, and then reprobed with other antibodies as indicated.

**RESULTS**

**Cbl Induces Ub Conjugation to TCRζ Chain**—Previous studies have shown that the TCR components including the TCRζ chain undergo activation-dependent ubiquitination (10, 11). The present study was made to determine the molecular events underlying Ub conjugation to TCRζ. As the first step, we tried to establish an in vivo system in which TCRζ can be properly ubiquitinated. For this purpose, TCRζ chain tagged with a Myc epitope at the C terminus was expressed in Jurkat T cells in the absence or presence of HA-tagged Ub. The cells were left untreated or were treated with pervanadate, a phosphatase inhibitor, or with both pervanadate and MG132, a proteasome inhibitor, and the cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates were immunoblotted with anti-HA. Coexpression of HA-Ub with TCRζ resulted in the formation of a slow migrating, higher molecular weight smear that was recognized by anti-HA antibody (Fig. 1a, top panel). Treatment of the cells with pervanadate increased Ub conjugation to TCRζ, and that was further enhanced by the presence of both pervanadate and MG132. To eliminate the interference of nonspecific co-precipitation of other proteins, we added 0.1% SDS in the lysis buffer. The same membrane was reprobed with anti-Myc antibody, showing equivalent amounts of TCRζ expression among all the samples (Fig. 1a, bottom panel).

The recent identification of Cbl as a RING-type E3 Ub-protein ligase (21) prompted us to examine whether Cbl can promote ubiquitination of TCRζ. Jurkat T cells were cotransfected with TCRζ and HA-Ub plasmas in the absence or presence of Cbl plasmid. Coexpression of Cbl with TCRζ significantly augmented Ub conjugation to TCRζ, which was further enhanced by pervanadate treatment (Fig. 1b). The results suggest that TCRζ can act as a substrate for Cbl E3 Ub ligase in T cells.

Previous studies have demonstrated that TCR cross-linking with OKT3 could also induce TCRζ ubiquitination (10, 11). To examine whether Ub conjugation to TCRζ could be induced under OKT3-stimulated conditions in our system, T cells transfected with TCRζ and Cbl were left unstimulated or stimulated with OKT3 or pervanadate. OKT3 stimulation indeed augmented Ub conjugation to TCRζ, although to a lesser degree than with pervanadate stimulation (Fig. 1c). The data were consistent with previous publications (10, 11), suggesting that TCRζ ubiquitination occurs under physiologic stimulation conditions.

**Cbl RING Finger and the Linker Region Are Required for Its Ligase Activity**—We have previously shown that mutation at cysteine 381 to alanine (C381A) of Cbl RING finger can disrupt the interaction of Cbl with UbcH7 and ablate its ligase activity in an in vitro system (21). To examine the effect of Cbl C381A mutant on TCRζ ubiquitination, we coexpressed TCRζ with wild-type Cbl or Cbl C381A mutant. Consistent with our in vitro study, the mutation at cysteine 381 to alanine abrogated Cbl-induced Ub conjugation to TCRζ under both resting and pervanadate-stimulated conditions (Fig. 2a).

A recent crystal structure study on Cbl-UbcH7 complex
The importance of the Cbl RING finger in Ub conjugation to TCR follows 360 b shows that besides the Cbl RING finger, which contains the primary binding sites for Ubc E2, a link region between the SH2 and RING domains is required for Cbl to promote TCRα ubiquitination.

**Fig. 1. Cbl promotes Ub conjugation to TCRα chain.** a, Jurkat T cells were transfected with 4 μg of plasmid containing a Myc-tagged TCRα cDNA together with 1 μg of either empty pEF vector or HA-tagged Ub plasmid by electroporation. After 48 h, cells were left unstimulated or stimulated with pervanadate (PV) with or without MG132 for 30 min. Cell lysates were incubated with anti-Myc, and the immunoprecipitates were subjected to immunoblotting with anti-HA antibody (top panel). The positions of polyubiquitinated TCRα protein (Ubαn-TCRα) are indicated. The same membrane was reprobed with anti-Myc antibody (bottom panel). The position of TCRα is indicated by an arrow. b, Jurkat T cells cotransfected with 4 μg of Myc-tagged TCRα plasmid plus 2 μg of either empty pEF vector or Cbl plasmid with or without 1 μg of HA-Ub plasmid as indicated. After 48 h, the cells were stimulated with pervanadate for 30 min and then lysed. The cell lysates were immunoprecipitated with anti-Myc, immunoblotted with anti-HA (top panel), and reprobed with anti-Myc (bottom panel). c, Jurkat T cells cotransfected with plasmids containing TCRα, Cbl, and HA-Ub were left unstimulated or stimulated with OKT3 or pervanadate for 30 min. The cell lysates were analyzed as described for panel b.

The results indicate that an intact RING finger and the linker region are required for Cbl to promote TCRα ubiquitination. Cbl N-terminal Variant SH2 Domain and the RING Finger Are Sufficient for TCRα Ubiquitination—Cbl consists of an N-terminal variant SH2 domain, a RING finger, and C-terminal proline-rich sequences (20). We next examined the functional requirement of each domain in Cbl for the Ub conjugation to TCRα. Truncated Cbl constructs containing Cbl variant SH2 domain and the RING finger (SH2+RING), or the RING finger and the C-terminal portion (RING+C) were generated and coexpressed with TCRα. The SH2+RING mutant induced Ub conjugation to TCRα to a similar degree as wild-type Cbl (Fig. 3a), under resting and pervanadate-stimulated conditions. However, the RING+C mutant showed markedly reduced ability to promote TCRα ubiquitination, in comparison with either wild-type Cbl or the SH2+RING mutant. We then tested whether the RING finger mutation at cysteine 381 to alanine in the SH2+RING mutant has an effect on TCRα ubiquitination. As compared with Cbl SH2+RING, the C381A mutant showed reduced ability in inducing Ub conjugation to TCRα (Fig. 3b). The data collectively suggest that the N-terminal SH2 domain and the RING finger are primarily responsible for TCRα ubiquitination.

**Fig. 2.** The role of Cbl RING finger and the linker region in TCRα ubiquitination. a, Jurkat T cells cotransfected with Myc-tagged TCRα and HA-Ub plasmids plus empty pEF vector, wild-type Cbl, or Cbl C381A mutant plasmid. After 48 h, cells were left unstimulated or stimulated with pervanadate (PV) for 30 min and then lysed. The cell lysates were immunoprecipitated with anti-Myc antibody, immunoblotted with anti-HA (top panel), and reprobed with anti-Myc (middle panel). The expression level of different Cbl mutants in the cell lysates was analyzed by immunoblotting the cell lysates with anti-Cbl antibody (bottom panel). b, Jurkat T cells were cotransfected with Myc-tagged TCRα and HA-Ub plasmids plus empty pEF vector, wild-type Cbl, Cbl E398A, or Cbl W408A mutant plasmid. After 48 h, the cells were analyzed as described for panel a.

The RING fingers of Cbl family proteins contain a well-conserved tryptophan residue (Trp-408), and mutation at this residue to alanine (W408A) reduces its binding to Ubc E2 and eliminates its ligase activity in vitro (21). To further confirm the importance of the Cbl RING finger in Ub conjugation to TCRα, we also coexpressed the W408A mutant with TCRα. In agreement with our in vitro data (21), the W408A mutant severely reduced TCRα ubiquitination under resting and pervanadate-treated conditions (Fig. 2b). Taken together, the results indicate that an intact RING finger and the linker region are required for Cbl to promote TCRα ubiquitination.
indirect complex with TCR\textgreek{z} and to transfer Ub to TCR\textgreek{z}. The importance of the Cbl N-terminal SH2 domain as demonstrated above may suggest that the putative adaptor molecule can interact with the variant SH2 domain and also with TCR\textgreek{z}. One of the potential molecules for this role is Zap-70, because it is the well characterized protein that binds to Cbl variant SH2 domain (20) and also to TCR\textgreek{z} (Zap-70, or \textgreek{z} chain-associated protein-70 as it was originally named) (1). We therefore tested the possibility of whether Zap-70 has any effect on TCR\textgreek{z} ubiquitination. Coexpression of Zap-70 with TCR\textgreek{z} showed a marked reduction in TCR\textgreek{z} ubiquitination under both resting and pervanadate-stimulated conditions. We then analyzed the effect of these mutations on the interaction with Zap-70. Jurkat T cells coexpressed with Zap-70 and the TCR\textgreek{z} mutants were left unstimulated or stimulated with pervanadate. The cell lysates were immunoprecipitated with anti-Myc antibody. The immunoprecipitates were immunoblotted with anti-Zap-70. Of all the TCR\textgreek{z} mutants, only the ABCYF mutant completely disrupted the binding to Zap-70 (Fig. 5b).

To further assess the role of TCR\textgreek{z} ITAMs in its ubiquitination, we examined the effect of the ABCYF mutant in the absence of Zap-70 or Cbl. The Zap-70-induced Ub conjugation to TCR\textgreek{z} was severely reduced in the ABCYF mutant (Fig. 5c). Similarly, the same mutation also reduced Cbl-mediated ubiquitination as compared with wild-type TCR\textgreek{z} (Fig. 5d). Collectively, the data suggest that any of three ITAMs on TCR\textgreek{z} can mediate Zap-70 interaction and the subsequent Cbl-mediated ubiquitination of TCR\textgreek{z}.

We then focused on the first ITAM of TCR\textgreek{z} to analyze its role in the Ub conjugation process. A previous study has shown that Ub conjugation to TCR\textgreek{z} chain occurs in multiple intracellular lysine residues (12). In the TCR\textgreek{z} 1–121 mutant, there are five lysine residues in the intracellular portion, whereas in the 1–121KR mutant, the last two lysine residues were mutated to arginines. We first examined whether the truncated mutants could be ubiquitinated. The 1–121 and the 1–121KR mutants displayed similar or slightly reduced Ub conjugation as compared with the full-length TCR\textgreek{z} under resting and pervanadate-stimulated conditions (Fig. 6a). Next, the effect of Cbl on their ubiquitination was analyzed by coexpressing Cbl and the full-length TCR\textgreek{z} or the truncated mutants. Under resting conditions, Cbl induced Ub conjugation to both the full-length TCR\textgreek{z} and the truncated mutants to a similar degree, although a slight reduction of ubiquitination in the truncated mutants was observed under pervanadate-stimulated conditions (Fig. 6b).

The role of the first ITAM on TCR\textgreek{z} was then analyzed by generating an AYF mutation in TCR\textgreek{z} 1–121 and by coexpressing them with Zap-70. The 1–121 AYF mutant displayed a significant reduction of Zap-70-induced Ub conjugation as compared with the unmutated TCR\textgreek{z} 1–121 under the same condi-
the cell lysates was analyzed by immunoblotting with anti-Zap-70 antibody (bottom panel). However, it was not known whether TCR

TCR 20). However, it was not known whether TCR

d with anti-Myc, and blotted with anti-HA. The positions of polyubiquitinated TCR

HA-Ub plasmids plus either empty pEF vector or Zap-70 plasmid. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA. The expression level of different Zap-70 mutants in

Zap-70, or Zap-70 Y292F mutant plasmid. After 48 h, the cells were treated, immunoprecipitated with Myc-tagged TCR

Cbl and Zap-70 were precipitated in an activation-dependent manner (Fig. 6d). Taken together, the results further support the notion that a single ITAM in TCRζ is sufficient to mediate its interaction with Zap-70 and its ubiquitination.

TCRζ, Zap-70, and Cbl Form a Macromolecular Complex—Previous studies have documented protein-protein interaction between TCRζ and Zap-70 and between Zap-70 and Cbl (1, 2, 20). However, it was not known whether TCRζ, Zap-70, and Cbl could form a single macromolecular complex. To address this possibility, we performed coimmunoprecipitation experiments in Jurkat T cells. Cell lysates from unstimulated or OIK3-stimulated Jurkat T cells were immunoprecipitated with anti-Cbl antibody. The immunoprecipitates were blotted with anti-TCRζ antibody and then with anti-Zap-70 antibody. As shown in Fig. 7a, both TCRζ and Zap-70 were coimmunoprecipitated by anti-Cbl antibody in an activation-dependent manner. Similarly, the cell lysates were immunoprecipitated with anti-TCRζ, and the immunoprecipitates were blotted with anti-Cbl and then with anti-Zap-70 antibody. Both Cbl and Zap-70 were co-precipitated by anti-TCRζ antibody (Fig. 7b). The endogenous formation of TCRζ-Zap-70-Cbl complex in Jurkat T cells prompted us to reconstitute such interaction in transiently transfected T cells overexpressing Myc-TCRζ and HA-Cbl. As shown in Fig. 7c, anti-HA antibody precipitated Myc-tagged TCRζ and endogenous Zap-70 in an activation-dependent manner. However, the TCRζ ABCYF mutant was not precipitated under the same conditions. Equal portions of the same cell lysates were also incubated with anti-Myc antibody. Cbl and Zap-70 were precipitated in an activation-dependent manner from cells transfected with wild-type TCRζ but not with the ABCYF mutant (Fig. 7d).

To further confirm that Zap-70 plays an adaptor’s role in

Cbl-Zap-70-TCRζ complex formation, we examined whether such complexes could also be formed in Zap-70-deficient P116 T cells. Consistent with Fig. 7b, anti-Cbl antibody precipitated TCRζ and Zap-70 from normal Jurkat T cells in an activation-dependent manner (Fig. 7e). However, under the same conditions, the amount of co-precipitated TCRζ was markedly reduced in P116 T cells, and no Zap-70 was detected in the same samples. The cell lysates from normal Jurkat T cells and P116 T cells were immunoblotted with anti-TCRζ, anti-Zap-70, and anti-Cbl antibodies. Equivalent amounts of TCRζ and Cbl were present in these two cell types, whereas Zap-70 was absent in P116 T cells (Fig. 7f). The results collectively suggest that TCRζ, Zap-70, and Cbl form macromolecular complexes in T cells, further supporting an adaptor’s role for Zap-70 in Cbl-induced Ub conjugation to TCRζ.

DISCUSSION

We and others have recently demonstrated that Cbl functions as an E3 Ub-protein ligase, with its RING finger recruiting Ub-loaded E2 conjugation enzymes and its variant SH2 domain binding to activated receptor tyrosine kinases for Ub conjugation (18, 21, 22). Numerous biochemical studies have documented an important role of Cbl in the immune system, particularly in T cell development and activation as a negative regulator (recently reviewed in Ref. 20). Consistent with this, Cbl gene-targeted mice display hyperplasia in the thymus, up-regulation of the expression of cell surface markers in thymocytes, and enhanced thymocyte activation (24, 25). However, a molecular link between Cbl E3 ligase activity and its negative regulation of T cell function remains to be elucidated. This study shows that Cbl acts as an E3 Ub ligase to promote ubiquitination of TCRζ chain, with the tyrosine kinase Zap-70 as an adaptor to bring together TCRζ-Zap-70-Cbl complex.
Jurkat T cells were cotransfected with Myc-tagged TCR-α loops. It provides a shallow groove formed by the AβYF (AYF) combination in the first ITAM or in combination (AYBF or ABCYF). After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The same membrane was reprobed with anti-Myc (bottom panel), b, Jurkat T cells were cotransfected with Zap-70 plasmid plus wild-type Myc-tagged TCR-ζ (Wt) or TCR-ζ mutant plasmids as indicated. After 48 h, the cells were left unstimulated or stimulated with pervanadate (PV) for 30 min, immunoprecipitated with anti-Myc, and blotted with anti-Zap-70 (top panel). The same membrane was reprobed with anti-Myc (middle panel). The expression level of Zap-70 in the cell lysates was analyzed by immunoblotting with anti-Zap-70 antibody (bottom panel). c, Jurkat T cells were cotransfected with Myc-tagged TCR-ζ or TCR-ζ ABCYF mutant plus HA-Ub and Zap-70 plasmids. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The same membrane was reprobed with anti-Myc (bottom panel). d, Jurkat T cells were cotransfected with Myc-tagged TCR-ζ or TCR-ζ ABCYF plus HA-Ub and Cbl plasmids. After 48 h, the cells were treated, immunoprecipitated, and blotted as described above.

The identification of Cbl RING finger to recruit E2 Ub conjugation enzyme to allow transfer of Ub to the substrate is further supported by a recent crystal structure study showing Cbl RING finger-UbcH7 E2 complex (23). The Cbl RING finger consists of a three-stranded β-sheet, an α-helix, and two large loops. It provides a shallow groove formed by the α-helix and the two zinc-chelating loops into which the tips of UbcH7 loops pack. Consistent with this structure, mutations at Cys-381 of the RING finger can disrupt Ubc E2 interaction and ablate its ligase activity (21, 22). Of note, in the RING finger-UbcH7 interface, tryptophan 408 of the RING finger forms multiple contacts with the residues on UbcH7, suggesting an essential role of this residue in Ubc E2 binding. Indeed, we previously showed that mutation of this residue to alanine reduces its role of this residue in Ubc E2 binding. Indeed, we previously showed that mutation of this residue to alanine reduces its ability for E2 binding and its E3 activities in an in vitro ubiquitination system (21). Here we provided in vivo evidence for the critical role of cysteine 381 and tryptophan 408 of Cbl RING finger, because mutation at either of these two sites abrogates or reduces Cbl-mediated Ub conjugation to TCR-ζ chain.

The crystal structure study also identified a linker region that connects the Cbl N-terminal variant SH2 domain and the RING finger (23). Besides its key structural role in Cbl itself, the linker region forms intermolecular hydrogen bond contacts with UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7. It was suggested that the involvement of this linker region in Ubc E2 binding might explain the fact that certain RING-type E3s may need more peptide sequences than UbcH7.

The effects of ITAMs on TCR-ζ ubiquitination. a, Jurkat T cells were cotransfected with HA-Ub plasmid plus either Myc-tagged TCR-ζ or plasmids containing TCR-ζ mutants in which the tyrosine (Y) was mutated to phenylalanine (F) in the first ITAM (AYF) or in combination (AYBF or ABCYF). After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The same membrane was reprobed with anti-Myc (bottom panel). b, Jurkat T cells were cotransfected with Zap-70 plasmid plus wild-type Myc-tagged TCR-ζ (Wt) or TCR-ζ mutant plasmids as indicated. After 48 h, the cells were left unstimulated or stimulated with pervanadate (PV) for 30 min, immunoprecipitated with anti-Myc, and blotted with anti-Zap-70 (top panel). The same membrane was reprobed with anti-Myc (middle panel). The expression level of Zap-70 in the cell lysates was analyzed by immunoblotting with anti-Zap-70 antibody (bottom panel).

The proximal ITAM of TCR-ζ can mediate its ubiquitination. a, Jurkat T cells were cotransfected with HA-Ub plasmid plus wild-type Myc-tagged TCR-ζ (Wt) or plasmids containing truncated TCR-ζ 1-121 or 1-121KR. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The same membrane was reprobed with anti-Myc (bottom panel). b, Jurkat T cells were cotransfected with HA-Ub plasmid plus Myc-tagged TCR-ζ (Wt), TCR-ζ 1-121, or TCR-ζ 1-121KR in the absence or presence of Cbl plasmid. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The membrane was reprobed with anti-Myc (bottom panel). c, Jurkat T cells were cotransfected with HA-Ub and Zap-70 plasmids plus either Myc-TCR-ζ 1-121 or TCR-ζ 1-121AYF mutant. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-HA (top panel). The membrane was reprobed with anti-Myc (bottom panel). d, Jurkat T cells were cotransfected with Zap-70 and Myc-tagged TCR-ζ 1-121 or TCR-ζ 1-121AYF. After 48 h, the cells were treated, immunoprecipitated with anti-Myc, and blotted with anti-Zap-70 (top panel). The same membrane was reprobed with anti-Myc (bottom panel).
immunoblotted with antibodies as indicated. Myc-TCRc, Jurkat T cells were transfected with HA-Cbl plasmid together with prepared from cells untreated or treated with pervanadate (top panel) were (bottom panel b). The same membrane was reprobed with anti-Zap-70 (middle panel). L indicates IgG light chain. The same membrane was then reprobed with anti-Cbl antibody (bottom panel). b, lysates prepared from cells untreated or treated with pervanadate (PV) were immunoprecipitated with anti-TCRζ antibody and blotted with anti-Cbl antibody (top panel), then with anti-Zap-70 antibody (middle panel). The same membrane was reprobed with anti-TCRζ (bottom panel). c, Jurkat T cells were transfected with HA-Cbl plasmid together with Myc-TCRζ (wt) or TCRζ ABCYF mutant. One-half of the cell lysates was subjected to immunoprecipitation with anti-HA antibody and immunoblotted with anti-Myc (top panel) or anti-Zap-70 (middle panel). The same membrane was reprobed with anti-HA (bottom panel). d, the other half of the same cell lysates was immunoprecipitated with anti-Myc antibody and blotted with anti-HA (top panel) or anti-Zap-70 (middle panel). The same membrane was reprobed with anti-Myc (bottom panel). e, Jurkat T cells and Zap-70-deficient P116 T cells were left unstimulated or stimulated with pervanadate. The cell lysates were immunoprecipitated with anti-Cbl and blotted with anti-TCRζ (top panel) and Zap-70 (middle panel). The same membrane was reprobed with anti-Cbl (bottom panel). f, aliquots of cell lysates from panel e were immunoblotted with antibodies as indicated.

Ubiquitination of TCRζ by Cbl E3 Ligase

Ubiquitination of TCRζ by Cbl E3 Ligase. Figure 7 shows a diagram of complex formation of TCRζ, Zap-70, and Cbl. a, Jurkat T cells were left unstimulated or stimulated with OKT3 for 5 min. The cell lysates were incubated with anti-Cbl antibody. The immunoprecipitates were blotted with anti-TCRζ antibody (top panel) and then with anti-Zap-70 antibody (middle panel). c, Jurkat T cells and Zap-70-deficient P116 T cells were left unstimulated or stimulated with pervanadate (PV) were immunoprecipitated with anti-TCRζ antibody and blotted with anti-Cbl antibody (top panel), then with anti-Zap-70 antibody (middle panel). e, Jurkat T cells and Zap-70-deficient P116 T cells were left unstimulated or stimulated with pervanadate. The cell lysates were immunoprecipitated with anti-Cbl and blotted with anti-TCRζ (top panel) and Zap-70 (middle panel). The same membrane was reprobed with anti-Cbl (bottom panel). g, aliquots of cell lysates from panel e were immunoblotted with antibodies as indicated.

showed that this tyrosine residue is in a buried environment not easily accessible for phosphorylation. Besides, it is not known at present whether this tyrosine residue is indeed phosphorylated and what could be the tyrosine kinase for the phosphorylation. This linker region more likely plays a structural role for Cbl to help keep Cbl variant SH2 domain and the RING finger, together with their binding proteins, in a proper conformation and/or orientation.

E3 ligases in the Ub system are responsible for recognition of the substrate and for the transfer of Ub to the substrate (13, 14). The present study demonstrates that the tyrosine kinase Zap-70 plays a previously unappreciated adaptor role for Cbl to recognize TCRζ and to help transfer Ub to TCRζ. This notion is supported by the following findings: 1) ectopic expression of Zap-70 with Cbl enhances Cbl-induced TCRζ ubiquitination; 2) disruption of the interaction between TCRζ and Zap-70, or between Zap-70 and Cbl, reduces Cbl-induced Ub conjugation to TCRζ; 3) Cbl SH2 + RING, which contains the Zap-70 binding site, is sufficient for TCRζ ubiquitination; and 4) TCRζ, Zap-70, and Cbl form macromolecular complexes in T cells. Therefore, in the TCRζ-Zap-70-Cbl complex, Zap-70 acts as a scaffold to bridge TCRζ, through an interaction of TCRζ ITAMs and the SH2 domains of Zap-70, to the vicinity of Cbl-Ubc E2 complex for Ub transfer, through an interaction of the negative regulatory tyrosine-292 in Zap-70 and the N-terminal variant SH2 domain of Cbl (30). Thus, the TCRζ-Zap-70 complex behaves like an intact receptor tyrosine kinase since the epidermal growth factor receptor, which provides a phosphotyrosine site for the docking of Cbl variant SH2 domain and also provides lysine residues for Ub conjugation.

We found that even with the Zap-70 Y292F mutant or the Cbl G306E mutant, which have been shown to disrupt the interaction between Zap-70 and Cbl (20), the TCRζ ubiquitination was still augmented (Fig. 4, b and c). The result raises a possibility that adaptor molecules other than Zap-70 may also facilitate the process. To further support this notion, we found that TCRζ does form a weak complex with Cbl in Zap-70-deficient P116 T cells (Fig. 7e). One candidate molecule in T cells could be the adaptor protein SLAP. Like Zap-70, SLAP disruption of the interaction between TCRζ and the N-terminal variant SH2 domain of Cbl (31, 32). Consistent with this idea, SLAP also negatively regulates TCR signaling (31). Obviously, further study is needed to examine whether SLAP can play an adaptor role similar to Zap-70 in Cbl-mediated TCRζ ubiquitination.

Analogous to the receptor tyrosine kinases, in which Cbl negatively regulates their function through ubiquitination and subsequent degradation (16, 18), previous studies have documented a negative role of Cbl in the regulation of Syk/Zap-70 family kinases (33–35). Cbl was shown to inhibit Syk activity in basophilic cells (33) or to induce the down-regulation of the protein expression level of Syk (34) and Zap-70 (35) in COS-1 cells. The data may suggest that Cbl could directly promote Ub conjugation to Syk and/or Zap-70. However, under the same conditions in which Cbl enhanced TCRζ ubiquitination, it induced Ub conjugation to Zap-70 to a much lesser degree.2 Although Cbl-induced inhibition of Zap-70 kinase activity and/or protein degradation could represent a direct role of Cbl on Zap-70, the present study supports an indirect model in which Zap-70 serves as an adaptor in Cbl-promoted ubiquitination of TCRζ chain. Should this be true, the enhanced activation of Zap-70 in Cbl-deficient thymocytes (24, 25) may also reflect the up-regulation of TCRζ by Cbl-mediated TCRζ ubiquitination.

2 H.-Y. Wang and Y.-C. Liu, unpublished data.
Lymphoid progenitors migrate from bone marrow to thymus and go through a series of defined stages of development to eventually generate peripheral T cell repertoire. During thymic development, these progenitors acquire various cell surface molecules such as TCR, CD4, and CD8. CD4 and CD8 double positive T cells then interact with the MHC expressed on antigen presenting cells to go through positive and negative selection processes (recently reviewed in Ref. 36). It is generally believed that the thymocytes receiving a moderate strength TCR signal are positively selected and develop further into mature T cells, whereas cells receiving signals that are either too weak or too strong will be eliminated by negative selection. By regulating the cell surface expression of TCR-CD3 components, Cbl may be involved in the fine tuning of the avidity and the strength of TCR/MHC interactions. Consistent with this, in Cbl-deficient mice, the MHC class II-restricted positive selection processes (recently reviewed in Ref. 36). It is generally believed that the thymocytes receiving a moderate strength TCR signal are positively selected and develop further into mature T cells, whereas cells receiving signals that are either too weak or too strong will be eliminated by negative selection.

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