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Deltex1 Promotes Protein Kinase C0 Degradation and Sustains Casitas B-Lineage Lymphoma Expression

Tzu-Sheng Hsu,*+† Huey-Wen Hsiao,† Pei-Jung Wu,*‡ Wen-Hsien Liu,† and Ming-Zong Lai*,+‡

The generation of T cell anergy is associated with upregulation of ubiquitin E3 ligases including Casitas B-lineage lymphoma (Cbl-b), Itch, gene related to anergy in lymphocyte, and deltex1 (DTX1). These E3 ligases attenuate T cell activation by targeting to signaling molecules. For example, Cbl-b and Itch promote the degradation of protein kinase C0 (PKC0) and phospholipase C-γ1 (PLC-γ1) in anergic Th1 cells. How these anergy-associated E3 ligases coordinate during T cell anergy remains largely unknown. In the current study, we found that PKC0 and PLC-γ1 are also downregulated by DTX1. DTX1 interacted with PKC0 and PLC-γ1 and stimulated the degradation of PKC0 and PLC-γ1. T cell anergy–induced proteolysis of PKC0 was prevented in Dtx1−/− T cells, supporting the essential role of DTX1 in PKC0 downregulation. Similar to Cbl-b and Itch, DTX1 promoted monoubiquitination of PKC0. Proteasome inhibitor did not inhibit DTX1-directed PKC0 degradation, but instead DTX1 directed the relocation of PKC0 into the lysosomal pathway. In addition, DTX1 interacted with Cbl-b and increased the protein levels of Cbl-b. We further demonstrated the possibility that, through the downregulation of PKC0, DTX1 prevented PKC0-induced Cbl-b degradation and increased Cbl-b protein stability. Our results suggest the coordination between E3 ligases during T cell anergy; DTX1 acts with Cbl-b to assure a more extensive silencing of PKC0, whereas DTX1-mediated PKC0 degradation further stabilizes Cbl-b. *The Journal of Immunology, 2014, 193: 000–000.

T cell anergy is one of the major mechanisms to maintain peripheral T cell tolerance. T cell anergy is induced by TCR engagement without appropriate costimulatory activation (1–4). T cell anergy could be generated in vitro by disproportional induction of calcium signaling and activation of NFAT (5, 6). Downstream of NFAT, several E3 ubiquitin ligases, including Casitas B-lineage lymphoma (Cbl-b), Itch, and the gene related to anergy in lymphocyte (GRAIL; ring finger protein 128), are upregulated in anergic T cells (2, 6–8). The physiological role of these E3 ligases in the regulation of T cell activation and the control of autoimmunity has been demonstrated in mice with genetic inactivation of each E3 ligase (9–13). One mechanism by which these anergy-inducible E3 ligases promote T cell anergy is by ubiquitination and/or degradation of T cell signaling molecules. For example, Cbl-b induces the ubiquitination of the p85 subunit of PI3K to prevent the recruitment of p85 by CD28 (14). Itch induces monoubiquitination of phospholipase C-γ (PLC-γ) to target the lysosome for degradation (7). Both Cbl-b and Itch promote protein kinase C0 (PKC0) degradation in anergic Th1 cells (7). Notably, there is a bidirectional interaction between Cbl-b and PKC0, as PKC0 also targets Cbl-b for ubiquitination and degradation (15). Many of the molecular processes involved in maintenance of T cell anergy by these E3 ligases remain incompletely understood. For example, only recently was the dispensable role of RING finger E3 ligase activity of Cbl-b in in vivo tolerance confirmed (16).

Deltex (DTX) is a target of Notch and composed of Notch-binding WEE domains at the N terminus, followed by a proline-rich motif and a C-terminal RING finger domain (17, 18). DTX1 confers ligand-independent activation of Notch by directing the ubiquitination and endosomal entry of Notch (19–21). We recently identified DTX1 as another target of NFAT and showed that it is induced during T cell anergy (22, 23). Knockout of DTX1 eliminates restriction in T cell activation, confers resistance to anergy induction, and results in generation of autoimmune syndromes in mice (23). DTX1 promotes the degradation of MEK kinase 1 and stimulates the expression of Cbl-b and GADD45B (23). In the current study, we found that DTX1 also targets PKC0 for degradation. Similar to Cbl-b, DTX1 interacted with PKC0. In Dtx1−/− T cells, anergy-induced downregulation of PKC0 was impaired. In addition, there was interaction between DTX1 and Cbl-b. DTX1 increased Cbl-b protein expression partly through antagonizing PKC0-mediated Cbl-b degradation. Our results suggest a novel crosstalk of DTX1 with Cbl-b in T cell anergy; both DTX1 and Cbl-b promote PKC0 downregulation, whereas DTX1 further enhances Cbl-b expression by preventing PKC0-directed Cbl-b proteolysis.

Materials and Methods

Reagents

Mouse DTX1 cDNA (18) was a gift from Dr. Hideyuki Okano (Keiko University, Tokyo, Japan). DTX1 mutants were generated as described previously (22, 23). Full-length PKC0 and Cbl-b cDNA was generated from mRNA of murine CD4+ T cell and cloned into pcDNA4-Myc-His-A

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Abbreviations used in this article: Cbl-b, Casitas B-lineage lymphoma; DTX1, deltex1; EGFP, enhanced GFP; GRAIL, gene related to anergy in lymphocyte; 3-MA, 3-methyladenine; PKC, protein kinase C; PLC-γ1, phospholipase C-γ1; PR, proline-rich; RF, RING finger; shRNA, short hairpin RNA; WCE, whole-cell extract; WT, wild-type.

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and pRES-hGFP-1α expression vector to yield Myc-tagged and Flag-tagged proteins. Calcium ionophore (A23187), MG132, 3-methyladenine (3-MA), and anti-Flag-HRP were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit anti-DTX1 polyclonal antisera was generated by raising Abs against GST-DTX1. The following Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA): PLC-γ1 (E-12), Zap-70 (485), SLP-76 (H-300), Lck (3A5), and Bcl-2 (G-1). Anti-Myc (9B11) was purchased from Cell Signalling Technology (Beverly, MA). Anti-PKClc (clone AA2) were purchased from Millipore (Temecula, CA). HRP-conjugated Pharmingen (San Diego, CA). Anti-actin (clone C4) and anti–mCherry, mOrange, mEGFP-DTX1, or mEGFP-DTX1 (proline-rich region 3A5), were purchased from Jackson ImmunoResearch Laboratories. WesternBright ECL HRP substrate was obtained from Advansta (Menlo Park, CA). DAPI-Fluoromount-G was obtained from Southern Biotechnology Associates (Birmingham, AL). The DTX1 mice were previously described (23). All mouse experiments were conducted under the approval of the Experimental Animal Committee, Academia Sinica.

Cell culture and transfection

T cells were cultured in RPMI 1640 medium supplemented with 10% FCS (both from Life Technologies), 10 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 20 mM 2-ME. DMEM (Life Technologies) was used in the culture of 293T cells. Transfection of 293T cells was performed by using OmicsFect In Vitro Transfection Reagent (Omics Biotechnology, Taiwan). Transfection of Jurkat T cells was conducted by electroporation on a Neon Transfection System (Invitrogen, Carlsbad, CA) at 1450 V, 30 ms, one pulse.

Th1 cells and anergy induction

Splenic CD4+ T cells (1 × 10^6/well) were activated with plate-bound 5 μg/ml anti-CD3 (2C11) and 2.5 μg/ml anti-CD28 (37.51) in the presence of anti-IL-4 (5 μg/ml, 11B10, BioLegend, San Diego, CA), mouse IL-12 (10 ng/ml; R&D Systems, Minneapolis, MN), and mouse IL-10 (12 ng/ml; R&D Systems) in 12-well plates for 5 days. Cultures were then washed and cultured in the presence of 10 ng/ml IL-2 for another 2 days. For anergy induction, Th1 cells were treated with 100 ng/ml A23187 for 16 h. IL-2 production was measured by [3H]thymidine incorporation into IL-2–dependent HT-2 cells. Anergy induction in Jurkat cells was achieved by treatment with A23187 (1 μg/ml).

Short hairpin RNA knockdown

Short hairpin RNA (shRNA) constructs containing target sequences were cloned into the pLentiLox vector (pL3.L7; gift from Dr. Luk Van Parijs, Massachusetts Institute of Technology, Cambridge, MA). The shRNA sequences were as follows: human DTX1, 5′-GAA CTT GAC CCG AAG AGG A-3′; human Cbl-b, 5′-ATC TTC AGT CAC ATG CTG GCA G-3′; human Itch, 5′-AAG TTG TTC TCA GAA TGA TGA-3′; and human PKCγ, 5′-GAG TAT GTC AAA GAA TGA GAG A-3′. Lentiviruses were harvested from HEK293T cells transfected with plL3.7 (12 μg), psPAX2 (9 μg), and pMD2.G (4 μg) for 48 h. Jurkat cells were infected with recombinant lentivirus, and GFP-expressing cells were isolated 48 h post-infection by sorting on an FACS Aria II (BD Biosciences). The human DTX1 sequence targeted by shRNA differs from the corresponding mouse DTX1 sequence by 9 nt.

Immunoprecipitation

Cells were washed twice with PBS buffer and lysed in whole-cell extract (WCE) buffer (25 mM HEPES [pH 7.4], 300 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA [pH 8], 0.1% Triton X-100, 0.5 mM DTT, and 1× complete protease inhibitor [EDTA-free; Roche]). For monoubiquitination detection, whole-cell extracts were incubated at 4°C for 1 h. Protein G Mag Sepharose (5 μl) was then added to the samples and incubated at 4°C for 1 h. The beads were washed repeatedly with 1 ml WCE buffer. The beads were mixed with 1× SDS-PAGE sample buffer and boiled at 100°C for 5 min. Polyvinylidene difluoride membranes (Millipore) were blocked with 5% skim milk for 1 h and probed with an appropriate Ab, followed by incubation with an HRP-conjugated secondary Ab. The protein bands were visualized by the ECL detection system.

Fluorescence protein expression

pmRFP-Rab5, pPSmOrange-tubulin, LAMP1-RFP, and pFPV-mCherry were obtained from Addgene. The enhanced (EGFP) coding fragment was subcloned into pcDNA4-DTX1-Myc to create the fusion construct pcDNA4-EGFP-DTX1. The mOrange coding fragment was subcloned into pcDNA4-PKClc-Myc to generate fusion protein construct pcDNA4-mOrange-PKClc. LAMP1 and mCherry encoding regions were cloned into pcDNA4-Myc-His-A to create a LAMP1 fusion protein construct pcDNA4-LAMP1-mCherry. Jurkat cells were transfected with mRFP-Rab5, LAMP1-mCherry, mOrange-PKClc, EGFP-DTX1, or EGFP-DTX1 (proline-rich region [3A5]). mOrange-positive cells were sorted on a FACS Aria II (BD Biosciences) 24 h later and then reseduced on 22 × 22 mm polysilane-coated glass coverslips and allowed to attach for another 2 h. Cells were fixed in 2% paraformaldehyde in PBS for 15 min at 37°C and permeabilized with 0.3% Triton X-100 for 10 min at room temperature. Cells were mounted in Dapi-Fluoromount-G (Southern Biotechnology Associates).

Image acquisition

Cells were observed under a Zeiss LSM 780 confocal microscope (Zeiss) with an objective lens of plan-apochromat 63×/1.4 Oil differential interference contrast at room temperature. EGFP fluorescence was excited by the Argon-Laser (488 nm), and fluorescence was collected in the range of 490 to 550 nm. mOrange fluorescence protein was excited by the DPSS-Laser (561 nm), and fluorescence was collected in the range of 565 to 580 nm. mRFP and mCherry fluorescence protein were excited by the HeNe-Laser (594 nm), and the emission was collected with 545 nm long-pass filter. DAPI-bound DNA was excited by the diode laser (405 nm), and fluorescence was collected in the range of 405 to 470 nm. The pinholes were as follows: Ch1-4.9 μm (blue), Ch2-10.1 μm (orange), Ch3-8.1 μm (green), and GaAsP-8.1 μm (red). Images were acquired by an AxioCam digital microscope camera (Zeiss) using Carl Zeiss software Zen 2010B SP1. Colocalization and intensity quantification were performed with intensity correlation coefficient using WCIF ImageJ software. Each individual cell was gated by ImageJ selection tool and Pearson correlation coefficient between mRFP (or mCherry) fluorescence was measured. A Pearson correlation coefficient value of 0 indicates nonoverlapping image, and a value of 1 represents 100% colocalization. Thirty individual cells were measured.

Results

DTX1 deficiency prevents PKC0 downregulation in anergic Jurkat cells

We examined the potential role of DTX1 in the expression of signaling proteins in anergic T cells. Jurkat T cells were treated with calcium ionophores to induce T cell anergy. A23187 priming led to downregulation of PKC0 in Jurkat cells (Fig. 1A), similar to a previous report in anergic Th1 cells (7). A decrease in PLC-γ1 in anergic Jurkat cells was also observed (Fig. 1). The levels of Zap70 and SLP76, in contrast, were not affected by anergy induction. Knockdown of DTX1 by specific shRNA in Jurkat cells prevented A23187-induced PKC0 degradation (Fig. 1A). Calcium ionophore–mediated PLC-γ1 downregulation was also reversed by DTX1 deficiency in Jurkat cells. To further confirm the specific role of DTX1, mouse DTX1 was reintroduced into DTX1-deficient Jurkat cells. Fig. 1B illustrates that reconstitution of DTX1 in DTX1-deficient Jurkat cells restored the sensitivity of PKC0 to A23187-triggered degradation. These results suggest that, analogous to the other two anergy-associated E3 ligases Cbl-b and Itch, DTX1 may also regulate the expression of PKC0 in anergic T cells. Notably, the expression levels of the PKC0 promoter, were increased in T cells treated with A23187 (Fig. 1B). Protein stability analysis illustrated that calcium signaling also increased the protein stability and phosphorylation analysis illustrated that calcium signaling also increased the protein stability and 1/2 of the transfected DTX1 in DTX1-deficient Jurkat cells (Supplemental Fig. 1). Therefore, in anergic T cell induced by calcium, DTX1 transcripts are induced by Ca2+-NFAT axis (23), whereas DTX1 proteins are further stabilized by calcium signaling.

DTX1 interacts with PKC0 and promotes PKC0 degradation

We examined whether DTX1 promoted PKC0 protein degradation. Coexpression of DTX1 with PKC0 led to reduction of PKC0 levels in 293T cells (Fig. 1C). DTX1 also decreased the expres-
sion of PLC-γ1 when cotransfected with PLC-γ1 into 293T cells (Supplemental Fig. 2A). Further, we found an association between DTX1 and PKCα (Fig. 2). In immunoprecipitation analysis, the Ab against DTX1-myc brought down PKCα. A DTX1–PLC-γ1 interaction was similarly found (Supplemental Fig. 2B). Because DTX1 exhibited similar effects on PKCα and PLC-γ1, only the inhibitory effects of DTX1 on PKCα were examined in detail in the current study. By using DTX1 mutants with deletion at the N-terminal two WWE domains (ΔN2), ΔPR, or the RING finger domain (ΔRF) (Fig. 2), we delineated the domains involved in such interaction in Jurkat cells. Both DTX1ΔN2 and DTX1ΔRF interacted with PKCθ in Jurkat cells, implying that either the WWE domains or RF domain is not involved in the binding of DTX1 to PKCθ (Fig. 2). In contrast, DTX1ΔPR did not bind PKCθ, suggesting that proline-rich domain is the PKCθ-binding region on DTX1. The functional role of DTX1 PR domain was also illustrated by that reconstitution of DTX1ΔPR failed to restore the ability of A23187 to downregulate PKCα in DTX1-FIGURE 1. DTX1 promotes PKCα downregulation. (A) DTX1 knockdown prevents PKCα downregulation in anergic Jurkat cells. Control (pLL3.7) and DTX1-knockdown (shDTX1) Jurkat cells were treated without or with A23187 (1 μg/ml) for 24 h to induce anergy. The levels of DTX1, PKCα, PLCγ1, Zap-70, SLP-76, and Lck were determined by immunoblotting. (B) Reintroduction of DTX1 restores anergy-induced PKCα downregulation. Jurkat-shDTX1 cells were transfected with or without mouse DTX1-Myc by electroporation. After 24 h, cells were treated with A23187 (1 μg/ml) for another 16 h. The levels of PKCα, DTX1-Myc, and Cbl-b were determined. (C) Coexpression of PKCα with DTX1 leads to PKCα degradation. 293T cells were transfected with PKCα-Myc and increasing amounts of DTX1-Flag, as indicated, and the levels of DTX1 and PKCα determined 48 h after transfection. (D) Failure of DTX1ΔPR to restore PKCα downregulation in anergic T cells. DTX1ΔPR–Myc was transfected into Jurkat-shDTX1 cells as described in (B). T cells were treated with A23187 and the levels of PKCα and DTX1-Myc determined. Experiments in (C) and (A), (B), and (D) were independently performed six times and three times, respectively, with similar results.

FIGURE 2. Interaction between DTX1 and PKCθ in T cells. Left panel illustrates the constructs of the full-length (FL) DTX1, DTX1 with RING finger deleted (ΔRF), DTX1 with the N-terminal two WWE domains truncated (ΔN2), and DTX1 with ΔPR deleted (ΔPR). Jurkat T cells were transfected with DTX1(FL)-Myc, DTX1ΔRF-Myc, DTX1ΔN-Myc, DTX1-RF-Myc, and PKCθ-Flag by electroporation. After 24 h, cells were treated with A23187 (0.5 μg/ml) for another 2 h. Cell lysates were prepared and precipitated with anti-Myc. The levels of PKCθ-Flag and DTX1-Myc in the immunoprecipitates and lysates were determined by anti-Flag and anti-Myc. Experiment was independently performed three times with similar results.
deficient Jurkat cells (Fig. 1D). For PKC\(\alpha\), the kinase domain–containing C terminus was found to interact with DTX1 (Supplemental Fig. 3). However, binding to DTX1 could also be detected in PKC\(\alpha\) kinase domain (Supplemental Fig. 3), suggesting the presence of additional region(s) on PKC\(\alpha\) that interact with DTX1.

**DTX1 promotes PKC\(\alpha\) degradation likely by the endosome–lysosome pathway**

We next examined how DTX1 promoted the downregulation of PKC\(\alpha\). PKC\(\alpha\) is subjected to monoubiquitination in anergic T cells (7), whereas DTX1 is known to promote Notch monoubiquitination (21). Fig. 3A illustrate that the monoubiquitination of PKC\(\alpha\) became prominent in Jurkat cells upon anergy induction by A23187 treatment. Coexpression of DTX1 with PKC\(\alpha\) also induced the monoubiquitination of PKC\(\alpha\) in 293T cells (Fig. 3B). We investigated whether DTX1 increased A23187-triggered PKC\(\alpha\) monoubiquitination. A23187-induced PKC\(\alpha\) monoubiquitination in Jurkat cells was further enhanced by the additional expression of DTX1, as well as by Cbl-b or Itch (Fig. 3C). To examine the contribution of the endogenous DTX1 to PKC\(\alpha\) monoubiquitination, we further used Jurkat cells deficient in DTX1, Cbl-b, or Itch (Supplemental Fig. 4A). Downregulation of DTX1 reduced monoubiquitination of PKC\(\alpha\) in A23187-treated Jurkat cells, in extents comparable to those observed by knockdown of either Cbl-b or Itch (Fig. 3D). Consistent with the decrease in PKC\(\alpha\) monoubiquitination, A23187-induced PKC\(\alpha\) degradation was largely prevented by downregulation of DTX1, Cbl-b, or Itch in Jurkat cells (Fig. 3D). We also found that DTX1-mediated PKC\(\alpha\) degradation was not inhibited by inclusion of MG132 (Fig. 3E), suggesting a process that is proteasome independent. Addition of 3-MA, an inhibitor of autophagy, did not interfere with DTX1-directed PKC\(\alpha\) degradation either (Fig. 3E). In contrast, the inclusion of NH\(_4\)Cl or chloroquine, inhibitors of endosome and lysosome

**FIGURE 3.** DTX1 is involved in PKC\(\alpha\) monoubiquitination and promotes PKC\(\alpha\) degradation in endosome–lysosome pathway. (A) Induction of T cell anergy triggers PKC\(\alpha\) monoubiquitination. Jurkat cells with hemagglutinin (HA)–ubiquitin constitutively expressed were treated with A23187 (1 \(\mu\)g/ml) for the indicated time points and cell lysates prepared. PKC\(\alpha\) ubiquitination was examined by immunoprecipitation (IP) of cell lysates with anti-HA and immunoblotting with anti-PKC\(\alpha\). (B) DTX1 promotes PKC\(\alpha\) monoubiquitination. PKC\(\alpha\)-Myc, DTX1-Flag, and/or HA-ubiquitin (Ub) were transfected into 293T cells, as indicated. After 24 h, cells were transfected with 20 \(\mu\)M MG132 for 6 h and cell lysates prepared. Cell lysates were immunoprecipitated with anti-HA and detected with anti-PKC\(\alpha\). Expression of DTX1 in lysates was determined with anti-Flag. (C) DTX1, Cbl-b, or Itch enhances A23187-induced PKC\(\alpha\) monoubiquitination in T cells. HA-ubiquitin–expressing Jurkat cells were transfected with DTX1-Myc, Cbl-b-Flag, or HA-Itch, as indicated. After 24 h, T cells were treated with A23187 for 4 h and WCE prepared. PKC\(\alpha\) ubiquitination was determined by immunoprecipitation with anti-HA and immunoblotting with anti-PKC\(\alpha\). The levels of the endogenous PKC\(\alpha\) and the transfected E3 ligases were determined by the respective Abs. (D) Knockdown of DTX1, Cbl-b, or Itch impaired T cell anergy–induced PKC\(\alpha\) monoubiquitination. Control (pL3.7), DTX1-knockdown (shDTX1), Cbl-b–knockdown (shCbl-b), or Itch-knockdown (shItch) Jurkat cells were transfected with HA-Ub. After 24 h, T cell anergy was induced by A23187 incubation for 4 h and WCE prepared. PKC\(\alpha\) ubiquitination and the endogenous PKC\(\alpha\) levels were determined as in (C). (E) DTX1 promotion of PKC\(\alpha\) degradation is proteasome independent. DTX1-Flag and/or PKC\(\alpha\)-Myc were transfected into HEK293 cells. After 24 h, cells were untreated for 6 h (Mock), treated with MG132 (20 \(\mu\)M) for 6 h, or 3-MA (10 mM) for 24 h. The levels of PKC\(\alpha\)-Flag and PKC\(\alpha\)-Myc were determined. (F) DTX1-induced PKC\(\alpha\) degradation was inhibited by NH\(_4\)Cl or chloroquine (CQ) in T cells. Jurkat cells were transfected with PKC\(\alpha\)-Myc and DTX1-Flag. After 12 h, cells were pretreated with NH\(_4\)Cl (20 mM) or CQ (25 mM) for 1 h, followed by A23187 treatment for 8 h. The levels of PKC\(\alpha\) and DTX1 were determined by anti-Myc and anti-Flag, respectively. Experiments in (A) and (C–F) and (B) were independently performed three times and four times, respectively, with similar results.
DTX1 is essential for downregulation of PKC\(\alpha\) in anergic primary Th1 cells

Previous studies have demonstrated that either Cbl-b or Itch is required for PKC\(\alpha\) degradation in anergic Th1 cells, as shown by the resistance of PKC\(\alpha\) to ionomycin-induced downregulation in Cblb\(^{-/-}\) or Itch\(^{-/-}\) primary Th1 cells (7). This raises a question of whether DTX1 is also required for the downregulation of PKC\(\alpha\) in anergic primary Th1 lymphocytes, given the involvement of Cbl-b and Itch in the same process. We therefore examined PKC\(\alpha\) degradation in anergic wild-type (WT) and Dtx1\(^{-/-}\) primary Th1 cells. Differentiated WT and Dtx1\(^{-/-}\) primary Th1 cells were treated with A23187 to induce T cell anergy. T cell anergy, shown by the attenuated Th1 cell proliferation upon restimulation, was impaired in Dtx1\(^{-/-}\) primary Th1 cells (Fig. 5A). As previously reported (7, 13), PKC\(\alpha\) levels remained unchanged in WT and Dtx1\(^{-/-}\) primary Th1 cells after A23187 treatment (Fig. 5B). Only after TCR ligation did the decrease of PKC\(\alpha\) become prominent in anergic primary Th1 cells (7), as shown in Fig. 5B. DTX1 deficiency prevented TCR-stimulated PKC\(\alpha\) downregulation in anergic primary Th1 cells (Fig. 5B). Therefore, DTX1 is also essential for PKC\(\alpha\) degradation in anergic normal T cells.

DTX1 interacts with Cbl-b and increases the stability of Cbl-b protein

We have previously demonstrated that DTX1 promotes the expression of Cblb mRNA (23). In Jurkat cells treated with A23187, knockdown of DTX1 impaired the induction of Cbl-b protein (Fig. 6A). Reintroduction of mouse DTX1 into DTX1-deficient Jurkat cells restored the expression of Cbl-b after A23187 treatment (Fig. 6A). We observed that coexpression of DTX1 with Cbl-b in 293T cells led to increased protein level of Cbl-b (Fig. 6B), suggesting that DTX1 also enhances the expression of Cbl-b at the protein level. Cbl-b protein stability was indeed decreased in anergic Jurkat cells (Fig. 6B). Cbl-b deficiency did not affect DTX1-mediated degradation of PKC\(\alpha\) in T cells (Fig. 6C), suggesting that DTX1 may induce PKC\(\alpha\) downregulation independently of Cbl-b.

We further studied whether there is an association between DTX1 and Cbl-b protein. In Jurkat T cells coexpressing DTX1–Myc and Cbl-b–Flag, precipitation with anti-Myc brought down Cbl-b (Fig. 7A). Truncation of the C-terminal RING finger or the N-terminal two WWE domains from DTX1 did not affect its interaction with Cbl-b, whereas the deletion of proline-rich region impaired the binding of DTX1 to Cbl-b in Jurkat cells (Fig. 7A), indicating that the proline-rich region is the Cbl-b–interacting segment. We also determined the DTX1-binding region on Cbl-b. Both N-terminal fragments and the middle regions of Cbl-b interacted with DTX1–Myc (Fig. 7B), suggesting the RF domain is the major DTX1-binding area of Cbl-b.
DTX1 protects Cbl-b from degradation

A similar result was greatly enhanced by PKC (Fig. 8B). In Jurkat cells, A23187-induced Cbl-b upregulation amounts of PKC (15). We found that coexpression of Cbl-b with increasing DTX1 interacted with PKC (Fig. 2) and bound Cbl-b (Fig. 7A). Together with the reported association between Cbl-b and PKC, the three-way interaction between DTX1, Cbl-b, and PKC was confirmed in anergic T cells (Fig. 8E). We found that DTX1 was constitutively associated with Cbl-b in untreated T cells (Fig. 8A), in contrast to the Cbl-b–PKC interaction detected after TCR/CD28 engagement or anergy induction (15) (Fig. 8E). The complex of DTX1–Cbl-b–PKC presumably could be formed after TCR activation in all T cells. However, DTX1 is immediately downregulated after TCR/CD28 engagement in regular T cell activation (22). We propose that only in anergic T cells is the upregulation of DTX1 sufficient for DTX1–Cbl-b–PKC complex formation and PKC degradation.

An unexpected finding is the upregulation of endogenous DTX1 in Cbl-b-knockdown T cells (Supplemental Fig. 4A). We attempted but have not identified the exact mechanism attributes to the enhanced expression of DTX1 caused by Cbl-b deficiency. However, this may help explain the observation that DTX1 triggered PKC degradation in Cbl-b-deficient T cells (Fig. 6C), as the excess DTX1 may compensate for the absence of Cbl-b, suggesting that the observed PKC degradation could be a con-
sequence of DTX1 overexpression. Whether DTX1 is capable of promoting PKC\v degradation in Cbl-b-independent manner in physiological condition remains to be determined.

Cbl-b, Itch, and DTX1 are involved in T cell anergy, but the cross-talk between these E3 ligases remain largely uncharacterized. Overexpressed Itch displays the ability to trigger the degradation of Cbl-b and DTX1 (30–32). Whether Itch is accessible to Cbl-b and DTX1 in anergic T cells and negatively regulates Cbl-b and DTX1 remains to be demonstrated. DTX1 has been shown to promote the expression of Cblb mRNA (23). In this study, we further found that DTX1 interacted with Cbl-b and enhanced the protein levels of Cbl-b. Our results therefore suggest the coordination between two E3 ligases in T cell anergy: both DTX1 and Cbl-b promotes PKC\v downregulation, whereas DTX1 further enhances Cbl-b protein expression by antagonizing PKC\v-mediated Cbl-b degradation.

Some of the T cell activation signaling are different between Dtx1\v/\v T cells and from Cblb\v/\v T cells. For instance, although the activation of ERK, JNK, and p38 MAPK is normal in Cblb\v/\v T cells (10, 33), it is greatly enhanced in Dtx1\v/\v T cells (23). Dtx1\v/\v T cells do not phenocopy Cblb\v/\v T cells, yet Dtx1\v/\v and Cblb\v/\v mice share some phenotype similarities (10, 11, 13, 23). T cells are overactive in Dtx1\v/\v T cells and Cblb\v/\v T cells. Dtx1\v/\v and Cblb\v/\v mice are also resistant to anergy induction. Further, spontaneous autoimmunity is observed in older Dtx1\v/\v and Cblb\v/\v mice. The suggestion of DTX1 as a partner that stabilizes Cbl-b protein in anergic T cells suggests

FIGURE 6. DTX1 increases the expression of Cbl-b. (A) Cbl-b is not upregulated in anergic DTX1-deficient Jurkat cells. Control (pLL3.7) and DTX1-knockdown (shDTX1) Jurkat cells were treated with A23187 (1 \mu g/ml) from 0 to 36 h. The levels of PKC\v, Cbl-b, and DTX1 were determined by immunoblotting. (B) Cotransfection of DTX1 enhances Cbl-b protein levels. Cbl-b was transfected into 293T cells with or without DTX1-Myc and the levels of Cbl-b determined. (C) Cbl-b knockdown did not affect DTX1-mediated PKC\v downregulation. Control and Cbl-b-knockdown Jurkat cells were transfected with DTX1 and PKC\v and the protein contents of DTX1 and PKC\v analyzed. Experiments in (A), (B), and (C) were independently performed three times, five times, and twice, respectively, with similar results.

FIGURE 7. DTX1 interacts with Cbl-b. (A) DTX1 interacts with Cbl-b through its PR regions. DTX1 and the indicated DTX1 mutants were transfected into Jurkat cells together with Cbl-b-Flag. After 24 h, cell lysates were prepared. Cell lysates were immunoprecipitated with anti-Myc, and the presence of Cbl-b, DTX1(FL), DTX1\vRF, DTX1\vN2, or DTX1\vPR in the precipitates and cell lysates was analyzed by anti-Flag and anti-Myc. (B) RF domain is the DTX1-binding region on Cbl-b. 293T cells were transfected with DTX1-Myc, Cbl-b(FL)-Flag, Cbl-b(FL)-Flag, Cbl-b(C-term)-Flag, or Cbl-b(Middle)-Flag. Cell lysate was immunoprecipitated by anti-Myc Ab. The presence of DTX1 and Cbl-b mutants in precipitates and cell lysates was determined. Experiments in (A) and (B) were independently performed two times with similar results.
that DTX1 contributes to some of the anergic role of Cbl-b in T cells. Recent studies reveal many regulatory functions of Cbl-b, including anergy induction in B cells and NKT cells, antitumor T cell immunity, and activation of mast cells and macrophages (34, 35). Whether DTX1 is also involved in the stabilization of Cbl-b in these functional activities deserves further investigation.

The deficiency of any one of Cbl-b, Itch (7), or DTX1 (Fig. 5B) affects anergy-induced degradation of PKCθ in Th1 cells. We further found that A23187-triggered PKCθ downregulation was impaired by DTX1 knockdown, Cbl-b knockdown, or Itch knockdown in Jurkat cells (Fig. 3D). This was correlated with a reduction in PKCθ monoubiquitination in anergic Jurkat cells deficient in DTX1, Cbl-b, or Itch (Fig. 3D). This was correlated with a reduction in PKCθ monoubiquitination in anergic Jurkat cells deficient in DTX1, Cbl-b, or Itch (Fig. 3D). The nonredundant role of Cbl-b, DTX1, and Itch in the proteolysis of PKCθ supports the notion that each E3 ligase acts through different essential mechanisms or participates in the different stages of the PKCθ proteolysis pathway in anergic T cells. Interestingly, DTX1 promotes Notch monoubiquitination and directs Notch into the early endosome (21), whereas the presence of Itch then relocalizes Notch to the multivesicular body for degradation (36), demonstrating the differential contribution of two E3 ligases to Notch downregulation along the endocytic processes. Whether DTX1, Cbl-b, and Itch may participate in the different stages of endosome–lysosome pathway in the proteolysis of PKCθ is currently being delineated.

In summary, we have found that DTX1, similar to Cbl-b and Itch, participates in T cell anergy-induced PKCθ degradation. In addition, there is a coupling of DTX1 to Cbl-b in T cell anergy.

DTX1 is constitutively associated with Cbl-b and T cell anergy-triggered additional recruitment of PKCθ, leading to PKCθ degradation. The present study thus suggests a new pathway in E3 ligase-mediated degradation of signaling molecules in anergic T cells. Our results also raise several questions that remain to be answered. A primary issue is on the exact molecular processes that DTX1, Cbl-b, and Itch participate in PKCθ degradation, as anergy-induced PKCθ degradation is compromised by deficiency in any one of the three E3 ligases. In addition, we have yet provided the direct evidences that DTX1–Cbl-b coordination in anergic T cell is operated in vivo. Further characterization will help resolve these issues for a clearer understanding of the molecular mechanisms underlying T cell anergy.

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Disclosures
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