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Selective Inhibition of FcεRI-Mediated Mast Cell Activation by a Truncated Variant of Cbl-b Related to the Rat Model of Type 1 Diabetes Mellitus

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Ubiquitin-protein ligase Cbl-b negatively regulates high affinity IgE receptor (FcεRI)–mediated degranulation and cytokine gene transcription in mast cells. In this study, we have examined the role of a truncated variant of Cbl-b related to the rat model of type 1 diabetes mellitus using the mast cell signaling model. Overexpression of the truncated Cbl-b that lacks the C-terminal region did not suppress the activation of proximal and distal signaling molecules leading to degranulation. FcεRI-mediated tyrosine phosphorylation of Syk, Gab2, and phospholipase C-γ1, and activation of c-Jun N-terminal kinase (JNK), extracellular signal–regulated kinase (ERK), p38 mitogen–activated protein kinase (MAP kinase), and inhibitor of nuclear factor κB kinase (IKK), and generation of Rac1 are unaffected in cells overexpressing the truncated Cbl-b in the lipid raft. On the other hand, FcεRI-mediated transcriptional activation of nuclear factor of activated T cells (NFAT), and transcription of interleukin-3 (IL-3) and IL-4 mRNA are inhibited by overexpression of the truncated variant of Cbl-b. This suppression parallels the re-compartmentalization of specific effector molecules in the lipid raft. These structural and functional analyses reveal the mechanism underlying the selective inhibition of cellular signaling by the truncated variant of Cbl-b related to insulin-dependent diabetes mellitus.

Key words: insulin-dependent diabetes mellitus, lipid raft, mast cells/basophils, protein-tyrosine phosphorylation, ubiquitin-protein ligase.

Abbreviations: FcεRI, high affinity IgE receptor; GEM, glycolipid-enriched microdomain; IDDM, insulin-dependent diabetes mellitus; NFAT, nuclear factor of activated T cells; pTyr, phosphotyrosine; SH2 domain, Src-homology-2 domain; UBA domain, ubiquitin-associated domain.

Mast cells play a central role in inflammatory and immediate allergic reactions. Recent genetic studies have revealed the signaling cascade from the high affinity IgE receptor (FcεRI) in mast cells (*1*). Aggregation of FcεRI triggers the activation of mast cells through the sequential activation of the non-receptor types of protein-tyrosine kinases Lyn and Syk. The activation of Lyn is regulated by the balance of its phosphorylation by protein-tyrosine phosphatase CD45, protein-tyrosine kinase Csk, and the adaptor protein Cbp (Csk-binding protein) (*2*). Subsequent activation of Syk is critical for FcεRI-mediated mast cell activation (*3*, *4*). Syk phosphorylates the adaptor protein LAT (linker for activation of T cells), which is localized in the lipid raft (also referred to as glycolipid-enriched microdomain: GEM). Phosphorylation of Tyr^{136} on LAT by Syk is required for association with PLC-γ1 (phospholipase C-γ1) (*5*). Syk also phosphorylates another adaptor protein, SLP-76 [SH2 (Src-homology-2)-containing leukocyte phosphoprotein of 76 kDa], which associates with Tec family protein-tyrosine kinase Btk. From the analogy with T cell receptor signaling, it is expected that LAT, SLP-76, and Gads (Grb2-related adaptor downstream of Shc) form a protein complex in the lipid raft leading to accumulation of the activated signaling molecules in mast cells (*6*). The lipid raft concentrates sphingolipids, cholesterol, glycophosphatidylinositol-linked proteins, and numerous activated signaling molecules in the plasma membrane. In addition to this Lyn-Syk-LAT pathway, there is another complementary signaling pathway initiated by Src-family kinase Fyn, which phosphorylates the adaptor protein Gab2 (Grb2-associated binder-2) (*7*). A genetic study has demonstrated that Gab2 is essential for FcεRI-mediated activation of PI-3 kinase (phosphatidylinositol 3-kinase), which generates PIP_3 [phosphatidylinositol-3,4,5-triphosphate: $PtdIns(3,4,5)P_3$] (*8*). Inhibition of FcεRI-mediated tyrosine phosphorylation of Gab2 by phenoxazine derivative Phx-1 suppresses IgE-mediated degranulation in mast cells (*9*). PIP_3 recruits the N-terminal PH (pleckstrin homology) domain of Btk to the plasma membrane. Btk associates with tyrosine-phosphorylated SLP-76 *via* the SH2 domain, and then becomes activated on phosphorylation of Tyr^{551} by Lyn. Activated Btk may contribute to both the phosphorylation and activation of PLC-γ1 in the

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plasma membrane. Crosstalk between the Lyn-Syk-LAT and complementary pathways is important for FcεRI-mediated activation of PLC-γ1, Ca^{2+} mobilization, degranulation, and cytokine production (*10*).

Cbl (Casitas B-cell lymphoma)-family ubiquitin-protein ligases c-Cbl and Cbl-b are involved in regulation of the immune system (*11*). Cellular protooncogene product c-Cbl acts as an E3 ubiquitin-protein ligase for activated protein-tyrosine kinases and c-Cbl–mediated ubiquitination leads to their degradation. Vaccinia virus–mediated overexpression of c-Cbl suppresses the antigen-induced activation of Syk and subsequent mast cell activation, suggesting that c-Cbl is a negative regulator of Syk in mast cells (*12*, *13*). We demonstrated that mast cells express Cbl-b in addition to c-Cbl (*14*, *15*). Antigen-stimulation induces tyrosine phosphorylation and translocation of Cbl-b to the lipid raft. Overexpression of Cbl-b in the lipid raft inhibits FcεRI-mediated degranulation and cytokine gene transcription through different mechanisms. Down-regulation of FcεRI-mediated degranulation requires the ubiquitin-protein ligase activity of Cbl-b whereas cytokine gene transcription is suppressed by overexpression of either the wild type or inactive form of Cbl-b in the lipid raft. Furthermore, overexpression of Cbl-b in the lipid raft suppresses tyrosine phosphorylation of FcεRI, Syk, and PLC-γ1, and dramatically down-regulates the protein amount of Gab2, suggesting that Cbl-b functions as a negative regulator of both the Lyn-Syk-LAT and complementary signaling pathways from FcεRI (*15*). The exact mechanism underlying Cbl-b–mediated down-regulation of cytokine gene transcription remains unclear. A genetic study revealed that Cbl-b deficiency in bone marrow–derived mast cells enhances FcεRI-induced tyrosine phosphorylation of Syk and PLC-γ1, the Ca^{2+} response and histamine release, supporting the idea that Cbl-b functions as a negative regulator of FcεRI-induced degranulation (*16*).

Cbl-b–deficient mice develop autoimmunity (*17*, *18*). A recent study highlighted the role of Cbl-b in T cell tolerance. Cbl-b is one of the anergy-inducing genes that may contribute to the antigen unresponsiveness of T cells (*19*). Upregulation of Cbl-b in anergic T cells promotes the degradation of PLC-γ1. Anergic T cells impair T cell receptor–mediated Ca^{2+} mobilization and are unable to maintain a mature immunological synapse, because Cbl-b is a negative regulator of receptor clustering and raft aggregation in T cells (*20*).

Recently, a truncated variant of Cbl-b was identified in the rat model of autoimmune disease type 1 diabetes mellitus (insulin-dependent diabetes mellitus: IDDM) (*21*). The mutant form of the rat *cbl-b* gene has a nonsense mutation that causes a lack of the C-terminal region of Cbl-b protein including the Pro-rich region and ubiquitin-associated domain (UBA domain) (*22*). Cbl-family ubiquitin-protein ligases require both the N-terminal SH2 domain and C-terminal UBA domain for negative regulation of immune receptor signaling. Therefore, the truncated form of Cbl-b is partially inactive and unable to associate with polyubiquitinated molecules that are required for the pathogenesis of type 1 diabetes through the C-terminal region of Cbl-b.

The present study has demonstrated the effect of overexpression of the truncated Cbl-b on FcεRI signaling. The

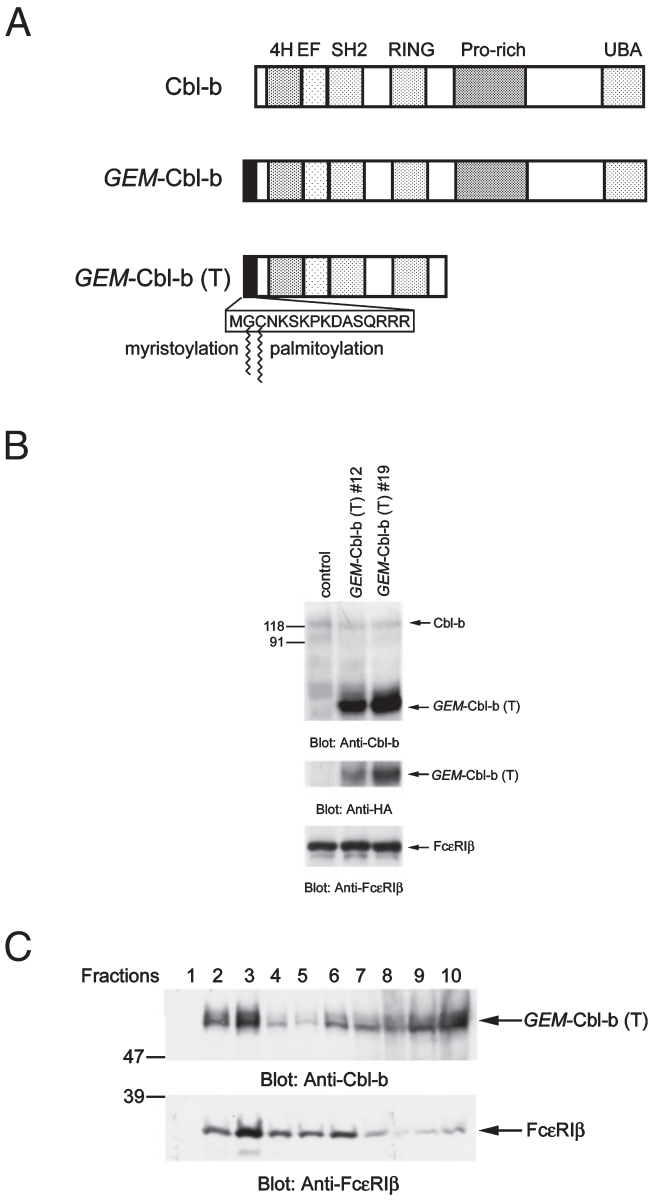

Fig. 1. **Generation of stable cell lines overexpressing the truncated variant of Cbl-b.** (A) Schematic diagrams of the wild type Cbl-b and lipid raft-targeted mutants. *GEM*-Cbl-b is a chimeric molecule of the myristoylation and palmitoylation signals joined to the N-terminus of Cbl-b. *GEM*-Cbl-b (T) lacks the Pro-rich region and UBA (ubiquitin-associated) domain (ΔSer^{484} to Leu^{982}). 4H indicates four-helix; EF, EF-hand Ca^{2+} binding domain; RING, RING finger domain; Pro-rich, proline-rich domain. (B) Generation of stable cell lines overexpressing *GEM*-Cbl-b (T). RBL-2H3 cells were stably transfected with pSVL-*GEM*-Cbl-b (T) and then selected with G418. Two positive cloned lines expressing the highest amounts of chimeric proteins were chosen for further analysis. Total cell lysates from RBL-2H3 and the selected cloned lines (clones #12 and #19) were analyzed by immunoblotting with anti–Cbl-b, anti-HA, and anti-FcεRIβ mAbs. Molecular size markers are indicated at the left in kilodaltons. (C) Constitutive localization of *GEM*-Cbl-b (T) in the lipid raft. RBL-2H3 cell homogenates (2×10^7) were fractionated by sucrose density gradient centrifugation. The proteins in each fraction were concentrated with 0.02% deoxycholic acid and 10% trichloroacetic acid, separated by SDS-PAGE, and then analyzed by immunoblotting with anti-HA and anti-FcεRIβ mAbs.

lipid raft–targeted, IDDM type of Cbl-b causes the selective dysfunction of Cbl-b to negatively regulate FcεRI-mediated cytokine production.

MATERIALS AND METHODS

Materials—Anti–Cbl-b, anti-Syk, anti–p38 MAP kinase, anti-IKK, and anti-Lyn antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti–hemagglutinin epitope (HA) mAb was obtained from Covance (Princeton, NJ, USA). Anti-dinitrophenyl IgE mAb (anti-DNP IgE, clone SPE-7), anti-tubulin mAb, and protein A–agarose beads were from Sigma (St Louis, MO, USA). Anti-phosphotyrosine (pTyr) (4G10), anti-Gab2, anti–PLC-γ1, anti-JNK/SAPK1, and anti-Rac1 antibodies were from Upstate Biotechnology (Lake Placid, NY, USA). Anti–phospho-p44/42 ERK (Thr^{202}/Tyr^{204}) (pERK) and anti-p44/42 ERK antibodies were from New England Biolabs (Beverly, MA, USA). Anti–phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) (pJNK), anti–phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) (p-p38), and anti–phospho-IKK (IKKα Ser^{180}/IKKβ Ser^{181}) (pIKK) antibodies were from Cell Signaling Technology (Beverly, MA, USA). Antigen 2,4-dinitrophenylated bovine serum albumin (DNP-BSA, 30 mol of DNP/1 mol of BSA) was from LSL (Tokyo, Japan). Anti-FcεRIβ mAbs were kindly provided by Dr. Juan Rivera (*23*) and Dr. Reuben P. Siraganian (National Institutes of Health, MD, USA).

Construction of DNA—The human Cbl-b cDNA was kindly provided by Dr. Stanley Lipkowitz (National Naval Medical Center, Bethesda, MD, USA). The cDNA encoding Cbl-b with the lipid raft localization signal was constructed by the PCR-based method (*15*). The cDNA of *GEM*-Cbl-b (T) was constructed by deletion of Ser^{484} to Leu^{982} of *GEM*-Cbl-b by PCR. A HA tag was fused to the C-terminal of Cbl-b constructs. The mutations were confirmed by DNA sequencing. The cDNAs were then subcloned into the pSVL expression vector (Amersham Biosciences, Piscataway, NJ, USA). Schematic diagram of the Cbl-b mutants used in this study is shown in Fig. 1A.

Cell Culture and Transfection—Rat basophilic leukemia RBL-2H3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM) (Sigma) containing 100 U/ml of penicillin and 10% heat-inactivated fetal calf serum. The stable cell lines overexpressing *GEM*-Cbl-b and *GEM*-Cbl-b (C373A) were described previously, and the magnitudes of the increase in the amount of Cbl-b on overexpression of the mutant forms of Cbl-b were 4-fold (*GEM*-Cbl-b, clone #12), 4-fold (*GEM*-Cbl-b, clone #18), 4-fold [*GEM*-Cbl-b (C373A), clone #4], and 3-fold [*GEM*-Cbl-b (C373A), clone #7], respectively (*15*). For stable transfection, 20 μg of linealized expression construct [pSVL-*GEM*-Cbl-b (T)] and 2 μg of pSV2-neo vector were cotransfected into 5×10^6 RBL-2H3 cells by electroporation (950 μF, 310 V). Cloned lines were selected with 0.4 mg/ml active G418 (Invitrogen, Carlsbad, California), and then screened as to the level of protein expression by immunoblotting of total cell lysates with anti–Cbl-b, anti-HA, and anti-FcεRIβ mAbs.

Subcellular Fractionation—Low-density detergent–insoluble fractions were prepared by sucrose density gradient centrifugation (*15*, *24*). Cells (2×10^7) stimulated without or with 30 ng/ml antigen DNP-BSA for 5 min were lysed in 2.4 ml of 0.05% Triton in MNEV buffer (150 mM NaCl, 25 mM Mes, pH 6.5, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) for 30 min on ice. Cell homogenates were mixed with equal volumes of 80% sucrose in MNEV buffer (final, 40% sucrose and 0.025% Triton), overlaid with 4.8 ml of 30% and 2.4 ml of 5% sucrose in MNEV buffer, and then centrifuged for 20 h at 200,000 × *g* (P40ST, Hitachi). Ten fractions were collected from the top of the gradient. The proteins were concentrated with 0.02% deoxycholic acid and 10% trichloroacetic acid. The proteins in fractions 2–4 were collected and utilized as the lipid raft fraction.

Cell Activation, Immunoprecipitation and Immunoblotting—Cell monolayers cultured overnight with anti-DNP IgE were washed once with Tyrode-Hepes buffer (10 mM Hepes, pH 7.4, 127 mM NaCl, 4 mM KCl, 0.5 mM KH_2PO_4, 1 mM $CaCl_2$, 0.6 mM $MgCl_2$, 10 mM $LiCl_2$, 5.6 mM glucose and 0.1% BSA) and then stimulated with 30 ng/ml antigen DNP-BSA in the same buffer for the indicated times (*25*, *26*). For the immunoprecipitation studies, the cells were solubilized in 1% Triton lysis buffer (1% Triton X-100, 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) on ice. In some experiments, the cells were solubilized in denaturation buffer (1% Triton X-100 lysis buffer containing 0.1% SDS and 0.5% deoxycholic acid) to dissociate protein complexes. Precleared cell lysates were incubated with the indicated antibodies prebound to protein A–agarose beads. After rotation for 1 h at 4°,C the beads were washed four times with the lysis buffer, and then the immunoprecipitated proteins were eluted by heat treatment at 100°C for 5 min with 2× sampling buffer. For the preparation of total cell lysates, the monolayers were lysed by the direct addition of 2× sampling buffer. Immunoprecipitated proteins and cell lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then electronically transferred to polyvinylidene difluoride transfer membranes (Millipore, Bedford, MA, USA). After blocking, the blots were reacted with the indicated primary antibodies and then horseradish peroxidase–conjugated secondary antibodies. Proteins were visualized with an enhanced chemiluminescence reagent (Western Lightning, PerkinElmer Life Sciences, Boston, MA, USA) (*27*, *28*). Densitometric analysis was carried out with NIH Image software.

Analysis of β-Hexosaminidase Release—Antigen-induced degranulation was determined by measurement of β-hexosaminidase release, as essentially described previously (*15*, *25*). Cells (2×10^5) were seeded into 24-well plates and cultured overnight with or without anti-DNP IgE. The cell monolayers were washed once with Tyrode-Hepes buffer and then stimulated with different concentrations of antigen DNP-BSA or 1 μM Ca^{2+} ionophore A23187 (Sigma) in the same buffer. After incubation for 1 h at 37°C, the medium was recovered and reacted with the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (Nacalai, Osaka, Japan). The release of the product 4-*p*-nitrophenol was monitored as the absorbance at 405 nm. The antigen-induced β-hexosaminidase release was expressed as a percentage of the release of β-hexosaminidase by 1 μM A23187.

Pull-Down Assay—The cDNA for human Pak1-PBD (p21-binding domain in serine/threonine kinase Pak1) (Lys^{67}–Ala^{150}) was isolated by RT-PCR from Jurkat T lymphocytes and then subcloned into the pGEX-4T.3 vector (Amersham) (*29*, *30*). Cells were solubilized in binding buffer (1% NP-40, 25 mM Hepes, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 2% glycerol, 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin) and cell lysates were precleared with 20 μg of GST (glutathione S-transferase) prebound to glutathione Sepharose 4B beads. After centrifugation, the resulting supernatants were reacted with 20 μg of GST-Pak1-PBD prebound to glutathione Sepharose 4B beads for 1 h at 4°C. The beads were washed four times with the binding buffer. Proteins interacting with Pak1-PBD were separated by SDS-PAGE and then analyzed by immunoblotting.

RNase Protection Assay—Antigen-induced transcription of multiple cytokine mRNAs was quantitatively measured with a Multi-probe RNase Protection Assay Kit (BD Biosciences, San Jose, CA, USA) (*15*). The total RNA extracted from non-stimulated or antigen-stimulated cells was hybridized with ^{32}P-labeled rat cytokine probes with a Riboquant In Vitro Transcription Kit (BD Biosciences). After the RNase treatment, the protected double-stranded RNAs were separated on a urea gel and then visualized by autoradiography.

Reporter Gene Assay—The NFAT reporter gene (5 μg) and Renilla-luc constructs (0.5 μg) were transiently cotransfected into RBL-2H3 cells or cells expressing the mutant forms of Cbl-b (10^7) by electroporation using a Nucleofector device (Amaxa GmbH, Cologne, Germany) (*29*). Thirty-six h after transfection, the cells were primed with anti-DNP IgE for further 12 h. Forty-eight hours after transfection, the cells were stimulated without or with 30 ng/ml antigen DNP-BSA for 6 h. The luciferase activity was determined with a Luciferase Assay System (Promega, Madison, WI, USA) using s luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany). The activity of the NFAT reporter gene (a gift from Dr. Gerald R. Crabtree, Stanford University, CA, USA) was normalized as to that of control Renilla-luc and expressed as the fold increase compared to the activity in non-stimulated cells. Aliquots of cell lysates were separated by SDS-PAGE and then analyzed by immunoblotting with anti-tubulin mAb as the internal control.

RESULTS

The C-Terminal Truncated Variant of Cbl-b Acts as a Dominant-Negative Mutant in FcεRI Signaling Leading to Degranulation—We previously demonstrated that the overexpression of Cbl-b in the lipid raft negatively regulates FcεRI-mediated mast cell activation (*15*). The lipid raft-associated Cbl-b acts as a dominant-active form in mast cell signaling. Thus, this system allows us to ana-

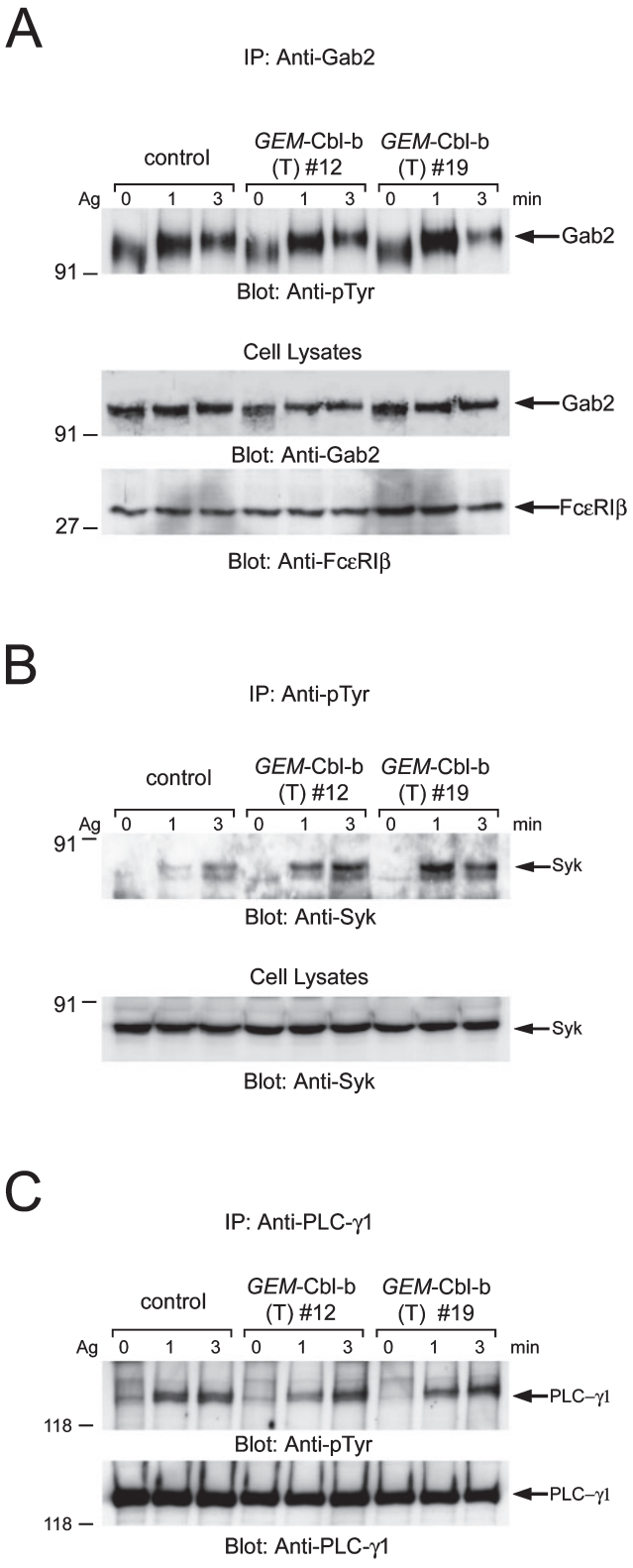

Fig. 2. **The C-terminal truncated variant of Cbl-b acts as a dominant-negative mutant for the proximal FcεRI signals.** Parental control cells and cells overexpressing *GEM*-Cbl-b (T) (clones #12 and #19) (5×10^6) were cultured overnight with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Cells were solubilized in the lysis buffer (A, C) or the denaturation buffer (B), and then immunoprecipitated with anti-Gab2 (A), anti-pTyr (B), or anti–PLC-γ1 antibodies (C), respectively. The immunoprecipitates and cell lysates were separated by SDS-PAGE and then analyzed by immunoblotting with the indicated antibodies. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

lyze the biological significance of Cbl-b. By using our established system, we attempted to determine the effect of overexpression of a truncated variant of Cbl-b that lacked the C-terminal Pro-rich region and UBA domain [Cbl-b (T)] on cellular function (Fig. 1A). On stable transfection, we chose two clones overexpressing Cbl-b (T) in the lipid raft [*GEM*-Cbl-b (T)]. Clone #19 expressed a higher amount of the truncated Cbl-b than clone #12 (Fig. 1B). Densitometric analysis demonstrated that the expression of *GEM*-Cbl-b (T) was 11-fold (clone #12) and 14-fold (clone #19) higher than that of endogenous Cbl-b. A subcellular fractionation study involving sucrose density gradient centrifugation demonstrated that *GEM*-Cbl-b (T) was localized in the lipid raft fraction of non-stimulated cells (Fig. 1C). This experiment allowed identification of the constitutive expression *GEM*-Cbl-b (T) in the lipid raft.

Genetic studies have revealed the essential signaling molecules for FcεRI-mediated mast cell activation. Previously, we demonstrated that the overexpression of Cbl-b in the lipid raft suppresses the antigen-induced tyrosine phosphorylation of Syk and Gab2, both of which play critical roles in the activation of PLC-γ1 (*15*). In particular, the expression level of Gab2 was dramatically suppressed by the lipid raft-targeted Cbl-b. Based on this knowledge, first we examined the effect of overexpression of Cbl-b (T) on the proximal signaling molecules by using these stable cell lines (Fig. 2). Overexpression of Cbl-b (T) in the lipid raft did not suppress the antigen-induced tyrosine phosphorylation of Gab2, Syk or PLC-γ1, compared to in the parental control cells. Moreover, tyrosine phosphorylation of Syk was relatively enhanced by overexpression of Cbl-b (T) in the lipid raft (Fig. 2B) suggesting that the missing region contributes to the negative regulation by Cbl-b in mast cells.

Next we examined the effect of overexpression of Cbl-b (T) on these distal signaling molecules (Fig. 3). Antigen-induced activation of JNK, ERK, p38 MAP kinase, and IKK was enhanced in the cells overexpressing Cbl-b (T) in the lipid raft. Furthermore, overexpression of Cbl-b (T) in the lipid raft resulted in enhancement of the antigen-induced degranulation (Fig. 4). Overall, this evidence clearly demonstrated that the C-terminal variant of Cbl-b acts as a dominant-negative mutant in FcεRI signaling leading to degranulation.

The C-Terminal Truncated Variant of Cbl-b Suppresses FcεRI-Mediated Activation of Transcription Factor and Cytokine Production—Cbl-b is known as a negative regulator of Vav1-Rac1–mediated cellular signaling including JNK activation (*31*). To elucidate the mechanism underlying Cbl-b–mediated suppression of cytokine gene transcription, we examined the effect of overexpression of Cbl-b in the lipid raft on the activation of Rac1. In this experiment, we compared the effect of *GEM*-Cbl-b (T)

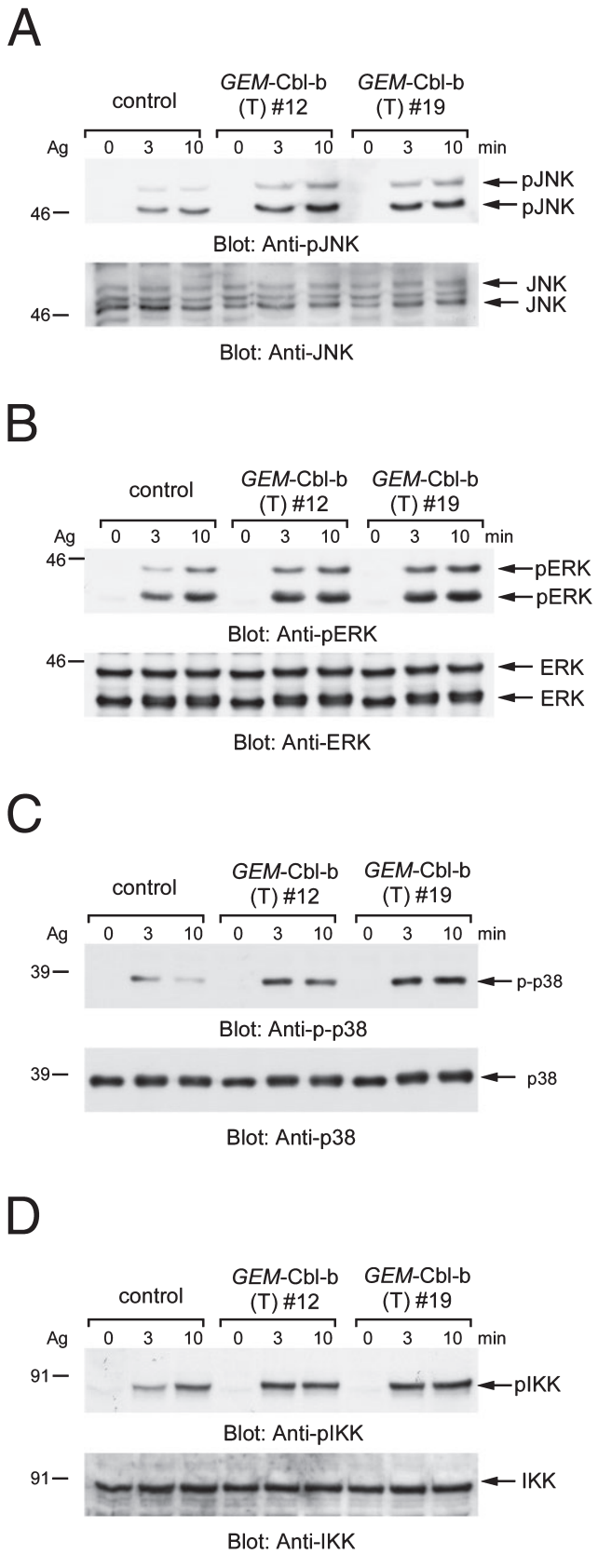

Fig. 3. **The C-terminal truncated variant of Cbl-b acts as a dominant-negative mutant in the activation of distal protein kinases.** Parental control cells and cells overexpressing *GEM*-Cbl-b (T) (clones #12 and #19) were cultured overnight with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Cell lysates ($1–2 \times 10^5$) were separated by SDS-PAGE, and then analyzed by immunoblotting with anti-pJNK and anti-JNK (A), anti-pERK and anti-ERK (B), anti–p-p38 MAP kinase and anti–p38 MAP kinase (C), or anti-pIKK and anti-IKK antibodies, respectively (D). Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

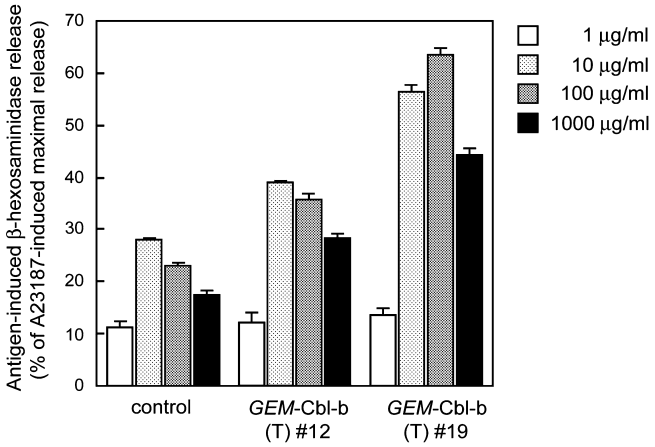

Fig. 4. **The C-terminal truncated variant of Cbl-b acts as a dominant-negative mutant in FcεRI-mediated β-hexosaminidase release.** Parental control cells and cells overexpressing *GEM*-Cbl-b (T) (clones #12 and #19) (2×10^5) were cultured overnight with anti-DNP IgE and then stimulated with the indicated concentrations of antigen DNP-BSA (Ag) (ng/ml). The normalized relative β-hexosaminidase release by the antigen is presented as a percentage of the control release by 1 μM Ca^{2+} ionophore A23187. The results are the mean values ± SD for three independent experiments.

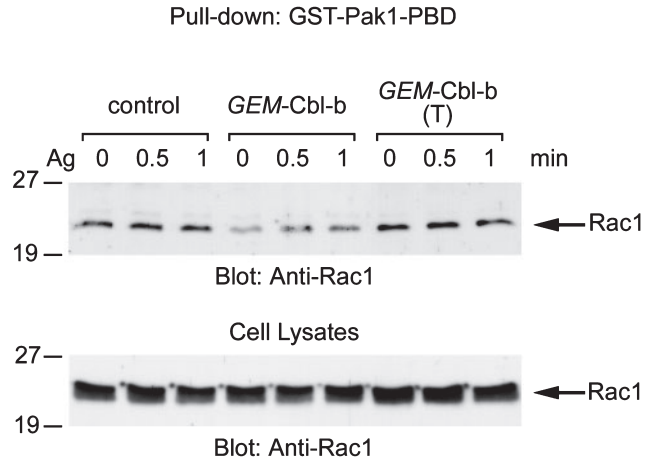

Fig. 5. **The C-terminal truncated variant of Cbl-b acts as a dominant-negative mutant in the activation of Rac1.** Pull-down assay. Parental control cells and cells overexpressing *GEM*-Cbl-b (clone #18) and *GEM*-Cbl-b (T) (clone #19) (3×10^6) were cultured overnight with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Cells were solubilized in the binding buffer and then precleared cell lysates were reacted with GST-Pak1-PBD prebound to glutathione Sepharose 4B beads. The bound proteins and cell lysates were analyzed by immunoblotting with anti-Rac1 antibodies. Molecular size markers are indicated at the left in kilodaltons. Similar results were obtained when the other cloned lines were examined.

using the cells overexpressing the wild type Cbl-b in the lipid raft (*GEM*-Cbl-b) used in our previous study (*15*). Densitometric analysis demonstrated that the antigen-stimulation resulted in a 44% increase in the generation of GTP-binding form of Rac1 in parental RBL-2H3 cells (Fig. 5) (*29*). Compared to the control RBL-2H3 cells, generation of the GTP binding form of Rac1 was impaired in the cells overexpressing wild type Cbl-b in the lipid raft.

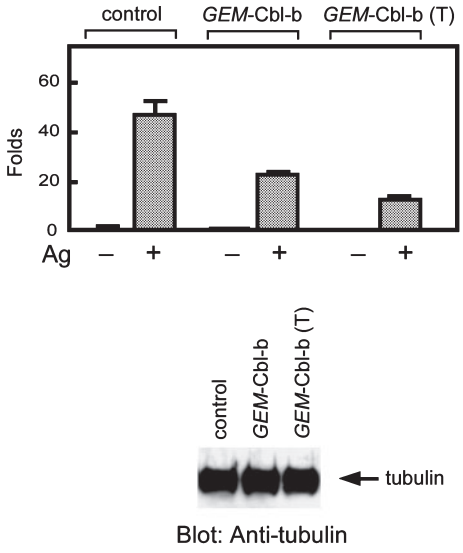

Fig. 6. **Overexpression of the C-terminal truncated variant of Cbl-b suppresses FcεRI-mediated activation NFAT.** The NFAT reporter gene construct was transiently transfected into parental control cells and cells overexpressing *GEM*-Cbl-b (clone #18) and *GEM*-Cbl-b (T) (clone #19) (10^7) by electroporation. Thirty-six hours after transfection, cells were cultured with anti-DNP IgE. Forty-eight h after transfection, the cells were stimulated without (–) or with (+) 30 ng/ml antigen DNP-BSA for 6 h. The normalized relative luciferase activities were expressed as fold increases compared to the activity in non-stimulated cells. Lower panel, aliquots of cell lysates were separated by SDS-PAGE and then analyzed by immunoblotting with anti-tubulin mAb. Similar results were obtained when the other cloned lines were examined.

However, overexpression of *GEM*-Cbl-b (T) did not suppress Rac1 activation. Thus, the C-terminal variant of Cbl-b acts as a dominant-negative mutant for Rac1 activation in FcεRI signaling.

Then we examined the effect of overexpression of Cbl-b (T) on the activation of transcription factors. Due to the transfection efficiency and the sensitivity limitation, we could only analyze the NFAT reporter gene. As expected, overexpression of the wild type Cbl-b in the lipid raft suppressed the antigen-induced activation of NFAT (Fig. 6). However, the overexpression of Cbl-b (T) in the lipid raft was capable of suppressing the activation of NFAT as well as the Cbl-b wild type. This suggested that the negative regulation of NFAT activation does not require the C-terminal region of Cbl-b. Alternatively, the N-terminal region and/or RING finger domain of Cbl-b in the lipid raft may down-regulate the activation of NFAT.

Analysis of the antigen-induced cytokine mRNA transcription supported this idea. Overexpression of Cbl-b (T) in the lipid raft inhibited the transcription of IL-3 and IL-4, but not tumor necrosis factor α (TNFα) mRNA (Fig. 7). Targeting of the C-terminal truncated form of Cbl-b to the lipid raft is sufficient for negative regulation of IL-3 and IL-4 production in mast cells.

Negative-Regulation of the Translocation of Specific Signaling Molecules to the Lipid Raft by Cbl-b—Previously, we demonstrated that Cbl-b negatively regulates the expression and tyrosine phosphorylation of Gab2 (*15*). Thus, we attempted to determine the localization of Gab2 in RBL-2H3 cells. Sucrose-density gradient centrifugation exper-

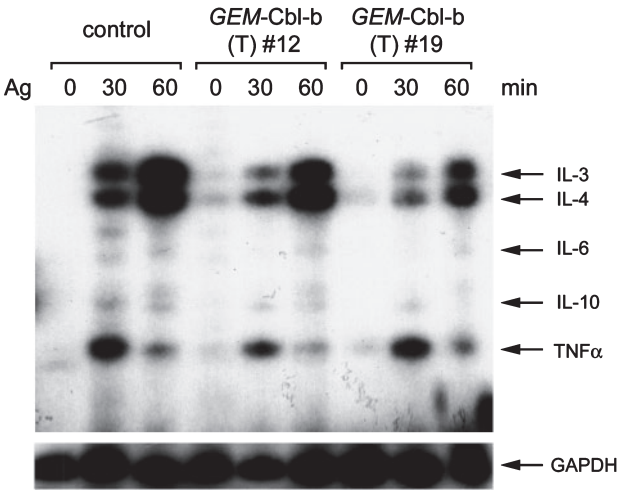

Fig. 7. **Overexpression of the C-terminal truncated variant of Cbl-b suppresses FcεRI-mediated IL-3 and IL-4 mRNA transcription.** Parental control cells and *GEM*-Cbl-b (T) overexpressing clones (clones #12 and #19) (5×10^6) were cultured overnight with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Total RNA was extracted and hybridized with the ^{32}P-labeled RNA probes of multiple rat cytokines. After RNase treatment, the protected double-stranded RNA was separated on a urea gel and analyzed by autoradiography. The results are representative of three independent experiments.

iments demonstrated that the adaptor protein Gab2 is localized in the lipid raft before and after stimulation. (Fig. 8A, top panels, fractions 2–4). A similar pattern was observed for the localization of PLC-γ1 (Fig. 8A, middle panels). Isolation of the lipid raft was confirmed by the localization of Lyn, which is constitutively targeted to the lipid raft by both myristoylation and palmitoylation signals (Fig. 8A, bottom panels).

Finally, we compared the localization of Gab2, PLC-γ1, and Syk among the control cells and the cells overexpressing the different kinds of Cbl-b in the lipid raft. Compared to the control cells, the localization of Gab2, PLC-γ1, and Syk in the lipid raft was strongly suppressed by overexpression of the lipid raft-targeted Cbl-b wild type in both unstimulated and antigen-stimulated cells (Fig. 8B). Moreover, the localization of Gab2 and PLC-γ1 in the lipid raft was dramatically suppressed by overexpression of the lipid raft-targeted ubiquitin-protein ligase inactive form of Cbl-b (C373A) or Cbl-b (T). On the other hand, the effect of these Cbl-b mutants on the localization of Syk was marginal. The isolation of the lipid raft from these cell lines was confirmed by immunoblotting with anti-Lyn and anti-FcεRIβ antibodies (Fig. 8B, bottom panels). Therefore, the negative regulation of cytokine (IL-3 and IL-4) production by Cbl-b could be explained by suppression of the lipid raft re-compartmentalization of Gab2, PLC-γ1, and Syk after antigen-stimulation.

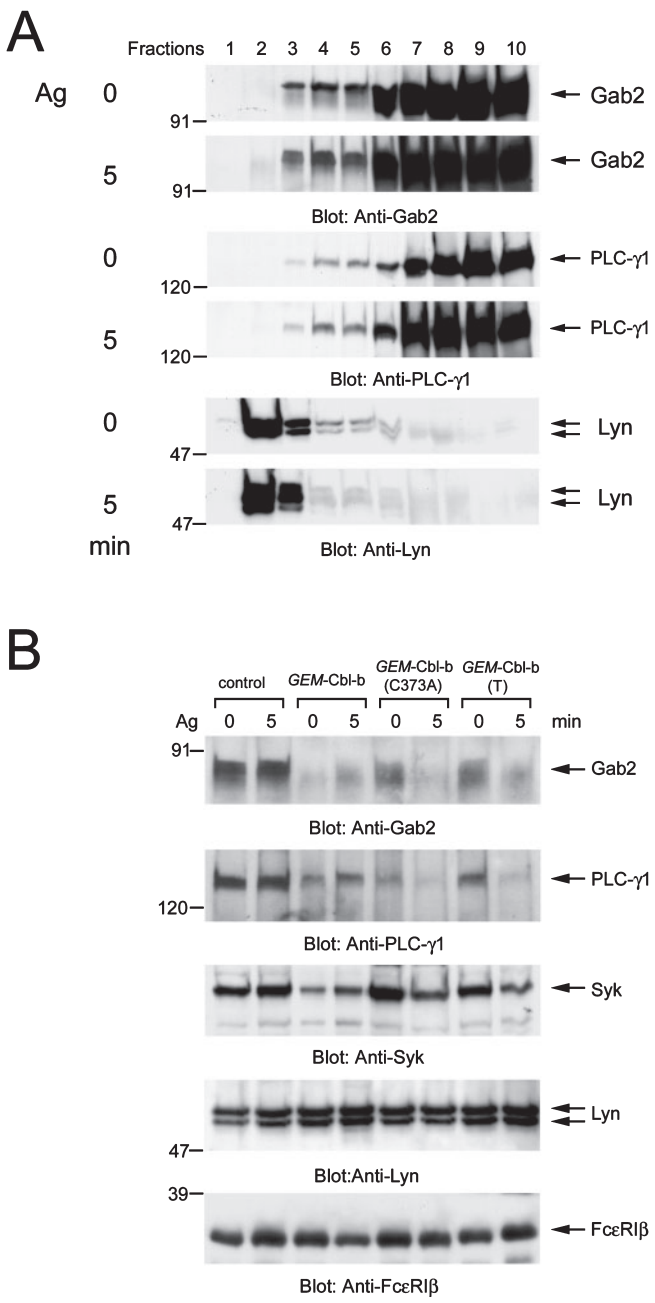

Fig. 8. **Cbl-b down-regulates the localization of signaling molecules in the lipid raft.** (A) Constitutive localization of Gab2 in the lipid raft. RBL-2H3 cells (2×10^7) were cultured overnight with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Cell homogenates were fractionated by sucrose density gradient centrifugation. The proteins in each fraction were concentrated with 0.02% deoxycholic acid and 10% trichloroacetic acid, separated by SDS-PAGE, and then analyzed by immunoblotting with anti-Gab2, anti–PLC-γ1, and anti-Lyn antibodies. The results are representative of three experiments. (B) Parental control cells and cells overexpressing *GEM*-Cbl-b (clone #18), *GEM*-Cbl-b (C373A) (clone #7), and *GEM*-Cbl-b (T) (clone #19) (2×10^7) were sensitized with anti-DNP IgE and then stimulated with 30 ng/ml antigen DNP-BSA (Ag) for the indicated times. Cell homogenates were fractionated by sucrose density gradient centrifugation. Proteins in the lipid raft fractions were collected, separated by SDS-PAGE, and then analyzed by immunoblotting with anti-Gab2, anti–PLC-γ1, anti-Syk, anti-Lyn, and anti-FcεRIβ antibodies. Molecular size markers are indicated at the left in kilodaltons. Similar results were obtained when the other cloned lines were examined.

DISCUSSION

The present study has demonstrated that the C-terminal region of Cbl-b plays a negative regulatory role in FcεRI-mediated cellular signaling leading to degranulation, but not the transcription of IL-3 and IL-4. There were two

reasons to focus on regarding the function of the C-terminal region of Cbl-b. First, comparison of the amino acid sequences of Cbl-b and c-Cbl revealed that the N-terminal region and RING finger domain are highly conserved in the two proteins, whereas the C-terminal region is not. This suggests that the C-terminal sequence could play roles that are unique to individual Cbl family members (*32*). Second, a deletion mutant of Cbl-b that lacks the C-terminal region was identified in the rat model of type 1 diabetes mellitus [Komeda diabetes-prone (KDP) rat]. This *cbl-b* gene mutation is responsible for the phenotype of KDP rats. The C-terminal region of Cbl-b has been thought to interact with the SH3 domains of cellular signaling molecules through the Pro-rich region. Also, Cbl-b is tyrosine phosphorylated through the engagement of FcεRI and may serve as a docking site for SH2 domain-containing molecules (*14*). Recently, the UBA domain was identified at the C-terminal end of Cbl family proteins. The UBA domain of Cbl-b interacts with ubiquitinated proteins and polyubiquitin chains in vitro, whereas the UBA domain of c-Cbl does not, suggesting that the difference in ubiquitin-binding may reflect the distinct functions of Cbl-b and c-Cbl. In fact, a genetic study demonstrated that Cbl-b and c-Cbl have different effects on FcεRI-mediated signal transduction and that Cbl-b, but not c-Cbl, functions as a negative regulator of FcεRI-induced degranulation (*16*).

Engagement of FcεRI induces a number of biochemical events leading to degranulation. The antigen-induced tyrosine phosphorylation of Gab2, Syk and PLC-γ1 is down-regulated by overexpression of Cbl-b in the lipid raft, depending on the ubiquitin-protein ligase activity or the C-terminal region-mediated function of Cbl-b (Fig. 2) (*15*). Crystal structural analysis demonstrated that the N-terminal region of c-Cbl is involved in the down-regulation of Syk family protein-tyrosine kinase ZAP-70 in T cells through interaction between phosphorylated Tyr^{292} in the linker region of ZAP-70 and the SH2 domain of c-Cbl (*33*). Since the N-terminal region of Cbl-b is highly homologous to that of c-Cbl (98%), it is expected that the N-terminal region of Cbl-b forms a complex with Syk through the association between phosphorylated Tyr^{316} in porcine Syk (corresponding to Tyr^{317} in rat and Tyr^{323} in human Syk, respectively) and Gly^{298} of Cbl-b in T cells (*34*, *35*). However, Pro^{296} in ZAP-70 (+4 position from Tyr^{292} that interacts with the deep pocket within the SH2 domain of c-Cbl) is not conserved in Syk family protein-tyrosine kinases and is replaced by Arg^{320} in porcine Syk (corresponding to Gly^{321} in rat and Leu^{327} in human Syk, respectively). In fact, we could not detect any interaction between Syk and the GST-N-terminal region of Cbl-b in a pull-down experiment using RBL-2H3 cells (data not shown). Therefore, although the N-terminal region of Cbl-b seems to be required for the interaction with the target molecules for protein-ubiquitination, the role of its interaction with the linker region of Syk remains speculative.

Genetic studies have demonstrated that thymocyte development is normal in Cbl-$b^{-/-}$ mice, whereas peripheral T-cell activation is affected (*17*, *18*). In addition, the lack of Cbl-b results in suppression of antigen-triggered receptor clustering, lipid raft aggregation, and sustained tyrosine phosphorylation of cellular proteins in T cells, *via* Vav1 activation (*20*). In the present study, we have demonstrated that the overexpression of Cbl-b in the lipid raft down-regulates the constitutive and inducible localization of Gab2 and PLC-γ1 in mast cells (Fig. 8B). The inhibition of the localization of Gab2 and PLC-γ1 is neither reversed on mutation of the RING finger domain (C373A) nor on deletion of the C-terminal region of Cbl-b. The suppression of the lipid raft localization seems to correlate with that of IL-3 and IL-4 transcription. This suggests that the localization before antigen-stimulation and the re-compartmentalization after the stimulation of effector molecules in the lipid raft are important for mast cell activation. This idea led us the idea that the lipid raft may be the site where Cbl-b recruits signaling molecules such as Gab2 and PLC-γ1, and affects their localization and re-compartmentalization in the lipid raft, and thereby down-regulates their activities, leading the negative signals to the cytokine synthesis. Alternatively, Cbl-b overexpression in the lipid raft strongly inhibits the effector activities in the complementary signaling pathway by decreasing both the localization and re-compartmentalization to the lipid raft, which is independent of the RING finger domain and the C-terminal region of Cbl-b. Presumably, the adaptive function of Cbl-b through the N-terminal region plays a negative regulatory role in the localization of specific signaling molecules in the lipid raft. On the other hand, the overexpression of Cbl-b mutants in the lipid raft partially suppresses the Lyn-Syk-LAT pathway by mainly blocking the re-compartmentalization of the effector molecule Syk. Therefore, our results suggest that Cbl-b regulates two signal pathways through different mechanisms, differently affecting the localization and re-compartmentalization of signaling molecules in the lipid raft. The effector molecules in the complementary pathway seem to be more sensitive to negative regulation by Cbl-b.

Previously, we reported that the antigen-induced tyrosine phosphorylation of Gab2, PLC-γ1, and Syk leading to degranulation are negatively regulated by the ubiquitin-protein ligase activity of Cbl-b (*15*). In this study, the overexpression of Cbl-b (T) in the lipid raft did not affect tyrosine phosphorylation of Gab2 and PLC-γ1, and enhanced Syk tyrosine phosphorylation, while it suppressed the re-compartmentation of these molecules in the lipid raft (Figs. 2 and 8). This suggested that tyrosine phosphorylation and the re-compartmentation of the early signaling molecules were not parallel events in FcεRI signaling. Tyrosine phosphorylation of activated signaling molecules was not sufficient for their translocation to the lipid raft. It is also possible that *GEM*-Cbl-b (T) promotes the protein degradation of the activated early signaling molecules in the lipid raft through ubiquitination. Therefore, these results also suggested that the RING finger domain and the C-terminal region of Cbl-b down-regulate the early FcεRI signaling through different mechanisms.

The present study demonstrated the selective inhibition of FcεRI-mediated mast cell activation by the truncated variant of Cbl-b related to the rat model of type 1 diabetes mellitus. This experimental system provided evidence that the IDDM type of Cbl-b partially functions in cellular signaling. This could explain the difference in the phenotype between the diabetic KDP rat and Cbl-$b^{-/-}$ mice, the latter of which exhibit severe autoimmune

disease. Since suppression of the transcription of some cytokine genes is RING-independent, the IDDM-type of Cbl-b may function as an adaptor in immune receptor signaling. Detailed analysis of the domain function of Cbl-b on FcεRI-mediated mast cells signaling could facilitate the development of a means of control of diabetes mellitus.

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