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Supplementary Materials for

PKC-θ Modulates the Strength of T Cell Responses by Targeting Cbl-b for Ubiquitination and Degradation

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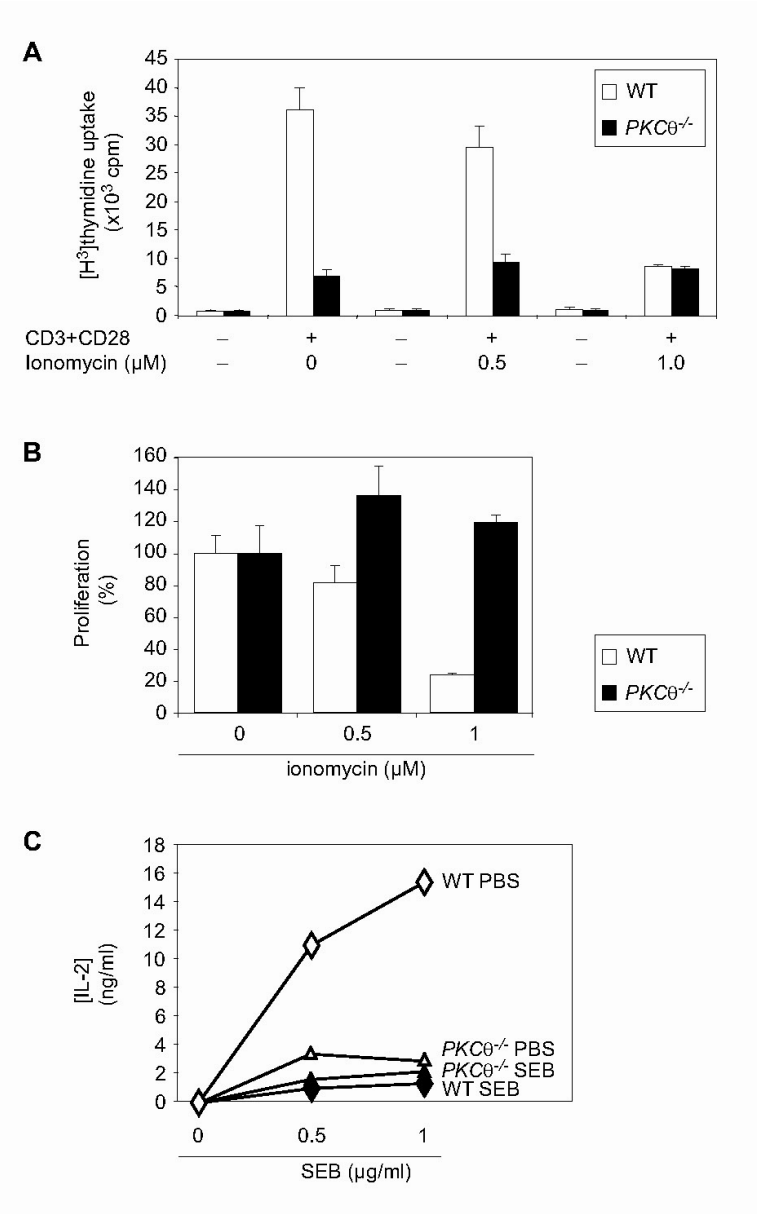

Fig. S1. *PKCθ*-deficient T cells exhibit hyporesponsiveness. (**A**) Anergy induced by pretreatment with Ca^{2+} ionophore (ionomycin) (*1*) inhibits the proliferation response in mouse $CD3^+$ T cells induced by stimulation of CD3 and CD28. Following washout, WT $CD3^+$ T cells in solvent alone responded strongly to stimulation of CD3 and CD28 but they were increasingly unresponsive as the ionomycin concentration was increased. In contrast, *PKCθ*-deficient $CD3^+$ cells already responded poorly to stimulation of CD3 and CD28 stimulation, and this hyporesponsiveness was not affected further by increasing concentrations of ionomycin. Of note, the defective proliferation of *PKCθ*$^{-/-}$ T cells can be mostly rescued by adding exogenous IL-2 (*2*). (**B**) The response of nontreated cells was set at 100%. *PKCθ*$^{-/-}$ $CD3^+$ cells already proliferated poorly prior to the induction of anergy; proliferation was not reduced further by ionomycin. All assays were performed in duplicate. Results shown are the means ± SD of three independent experiments. (**C**) Anergy induced by SEB in vivo inhibits the IL-2 response of ex vivo restimulated $CD4^+$ T cells. Cells from SEB-pretreated WT mice were anergized efficiently, whereas cells from *PKCθ*$^{-/-}$ mice already responded poorly after treatment with PBS, and this hyporesponsiveness was not further affected by pretreatment with SEB.

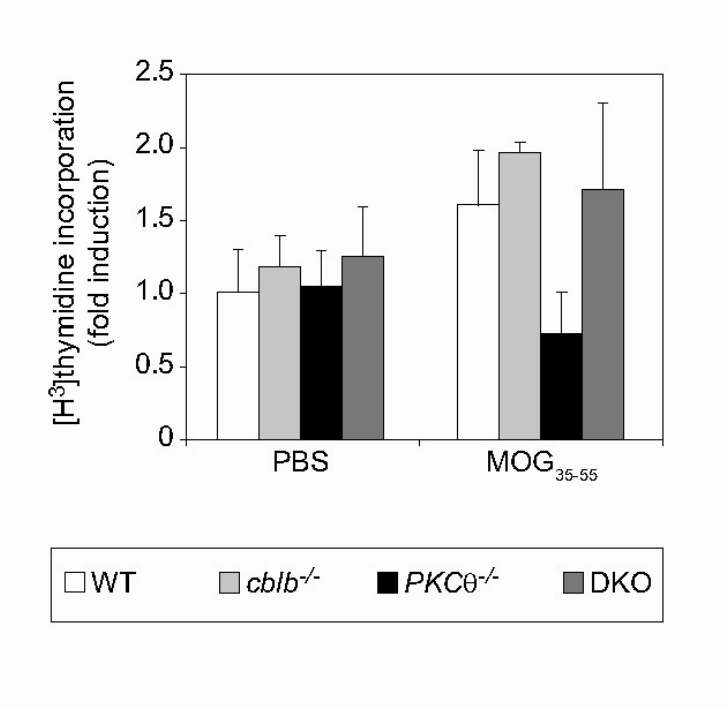

Fig. S2. The PKC-θ-dependent recall response is restored to WT levels in DKO T cells. Cell proliferation of *PKCθ*$^{-/-}$ T cells upon recall stimulation with 10 μg/ml of MOG_{35-55} peptide. Results shown are the means ± SD of one representative experiment.

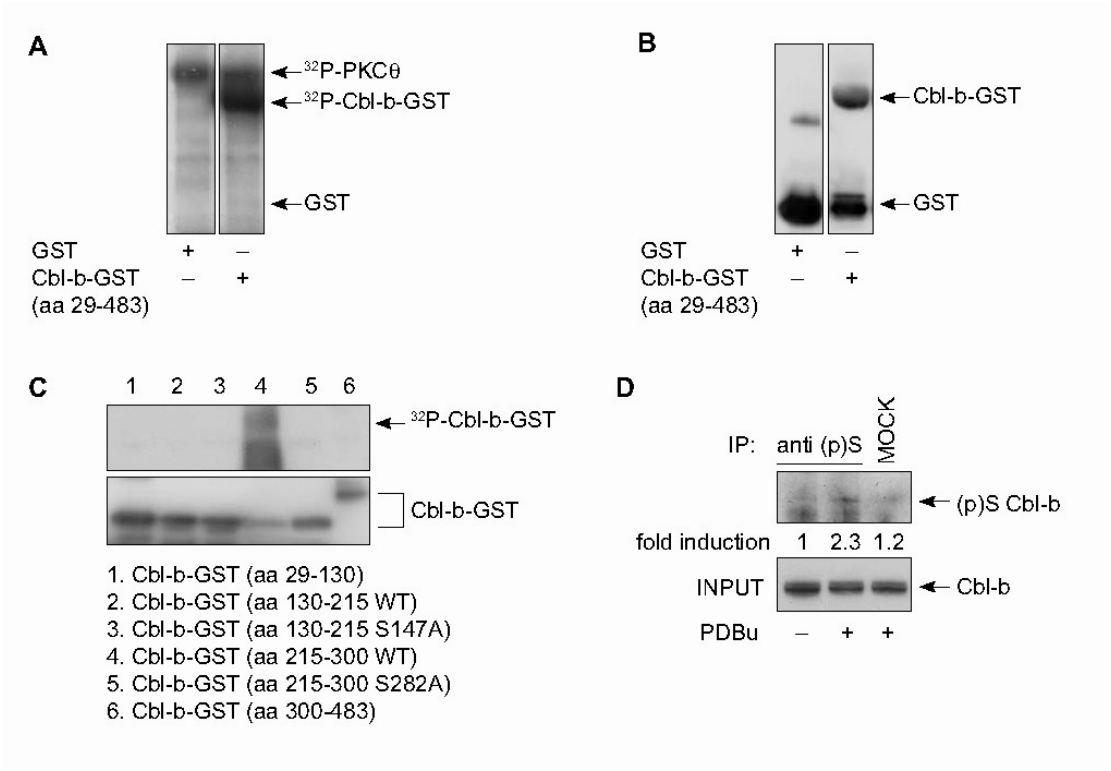

Fig. S3. PKC-θ phosphorylates the TKB domain of Cbl-b in vitro. (**A** to **C**) Kinase assay of GST-Cbl-b subdomains incubated with recombinant PKC-θ. Specific phosphorylation of the GST-Cbl-b TKB domain (N-terminal region of Cbl-b ranging from amino acid residues 29 to 483), and particularly of Ser^{282} on the GST-Cbl-b subdomain that ranged from residues 215 to 300 (lane 4) was reproducibly detected. In contrast, no phosphorylation of the S282A mutant fusion protein (lane 5) nor S147 (another bioinformatically identifed PKC site within the subdomain ranging from residues 130 to 215) or GST alone could be observed. Of note, however, upon long exposure, additional phosphorylation by PKC-θ could be observed in both the Cbl-b subdomains ranging from residues 29 to 130 and 300 to 483. These additional sites, however, remain unmapped. Analysis of Western blots with an anti-GST antibody confirmed equal loading (B, lower panel in C). Experiments were repeated at least two times, with similar results. (**D**) Serine-phosphostatus analysis of Cbl-b in intact T cells. Serine-phosphorylated Cbl-b was immunoprecipitated with a broadly reactive anti-pSer antibody. Stimulation of Jurkat T cells with PDBu (a pleiotropic PKC agonist) led to a 2.3-fold higher reactivity of this anti-pSer antibody with Cbl-b (upper panel). The input control for Cbl-b (bottom panel) is shown.

Table S1. Effects of single or double deficiencies in *cblb* and *PKCθ* on cellularity. Data shown are the means ± SEM (n = 3) of the percentage of cells positive for the indicated surface markers from splenocytes isolated from the indicated mice.

	WT	**cblb$^{-/-}$**	**PKCθ$^{-/-}$**	***DKO***
$CD3^+$	41.7 ± 8.2	32.2 ± 7.9	39.1 ± 3.3	30.7 ± 3.0
$CD4^+$	21.3 ± 0.7	17.7 ± 0.1	17.2 ± 0.1	13.6 ± 1.6
$CD8^+$	11.6 ± 0.7	10.2 ± 0.3	12.0 ± 0.7	8.6 ± 0.3
$V\beta8^+$	19.4 ± 2.2	20.7 ± 4.4	20.8 ± 2.8	14.8 ± 3.0

Table S2. Relative abundance of CD25, CD44, and CD69 on $CD3^+$ T cells by genotype. Flow cytometric analysis of $CD3^+$ T cells isolated from the indicated mice was performed on unstimulated cells and on cells after 16 hours of stimulation with antibodies against CD3 and CD28. Data shown are the mean fluorescent intensities ± SEM from 3 experiments.

	Unstimulated				**Stimulated**			
	WT	***cblb*$^{-/-}$**	***PKCθ*$^{/-}$**	**DKO**	**WT**	***cblb*$^{-/-}$**	***PKCθ*$^{/-}$**	**DKO**
CD25	3.6 ± 0.2	4.0 ± 0.3	3.7 ± 0.2	3.7 ± 0.4	242.2 ± 64.6	326.7 ± 106.5	143.4 ± 64.7	418.1 ± 175.8
CD44	59.6 ± 14.4	42.2 ± 19.9	46.3 ± 15.8	50.9 ± 10.7	150.6 ± 35.0	149.9 ± 48.7	90.1 ± 17.6	177.7 ± 24.2
CD69	4.0 ± 0.4	3.8 ± 0.3	4.1 ± 0.4	3.6 ± 0.4	208.9 ± 65.9	260.7 ± 97.3	143.8 ± 43.6	225.3 ± 45.1

Table S3. Specific interactions between PKC-θ and Cbl-b in the GAL4 yeast two-hybrid system. -, +, and ++ define the relative Leu-prototrophy of the yeast clones as a readout of protein-protein interactions.

DNA-binding domain (bait)	Activation domain hybrid (prey)	Leu-prototrophy
PKC-θ N-terminus	Control	-
PKC-θ C-terminus	Control	-
PKC-θ C2-like domain	Control	-
PKC-α N-terminus	Control	-
PKC-θ N-terminus	Cbl-b WT	+
PKC-θ C-terminus	Cbl-b WT	-
PKC-θ C2-like domain	Cbl-b WT	-
PKC-α N-terminus	Cbl-b WT	++
PKC-θ N-terminus	Cbl-b N-terminus	+
PKC-θ C-terminus	Cbl-b N-terminus	-
PKC-θ C2-like domain	Cbl-b N-terminus	-
PKC-α N-terminus	Cbl-b N-terminus	++
Control	Control	-
Control	Control	-
Control	Control	-
Control	Control	-

References

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