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Cbp Deficiency Alters Csk Localization in Lipid Rafts but Does Not Affect T-Cell Development

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The ubiquitously expressed transmembrane adaptor Csk-binding protein (Cbp) recruits Csk to lipid rafts, where the latter exerts its negative regulatory effect on the Src family of protein tyrosine kinases. We have inactivated Cbp in the mouse germ line. In contrast to *Csk* gene inactivation, which leads to embryonic lethality and impaired T-cell development, Cbp-deficient mice were viable and exhibited normal T-cell development but with an increased thymocyte population. In the absence of Cbp, the amount of Csk that localizes to the lipid rafts was greatly reduced. Interestingly, this altered lipid raft localization of Csk did not lead to any detectable biochemical or functional defect in T cells. The T-cell receptor-induced intracellular calcium flux, cell proliferation, and cytokine secretion were not affected by the absence of Cbp. Peripheral T-cell tolerance to superantigen SEB was also largely intact in Cbp-deficient mice. Thus, Cbp is dispensable for T-cell development and activation.

T-cell receptor (TCR) signaling is essential for the differentiation and activation of T lymphocytes. It is initiated upon phosphorylation of the tyrosine residues found in the immunoreceptor tyrosine-based activation motifs of the TCR-associated invariant CD3 polypeptide chains by the Src family of protein tyrosine kinases (PTKs). Two of these PTKs, Lck and Fyn, in particular, have been shown to play critical roles in TCR signaling (4, 14, 22).

In turn, the activity of the Src family of PTKs is modulated by the phosphorylation status of their inhibitory carboxyl-terminal tyrosine residue, which in $pp60^{c\text{-}src}$ corresponds to tyrosine 527 of the kinase (6, 7). The phosphorylation of this inhibitory tyrosine residue is accomplished by the carboxyl-terminal Src kinase (Csk) and leads to an intramolecular interaction of this phosphorylated tyrosine with the SH2 domain of the Src family of PTKs. This results in a conformational change that represses the kinase activities of the Src family of PTKs (26, 30). The importance of Csk is evidenced by its genetic ablation in mouse, which leads to an early embryonic-lethal phenotype due to a neural developmental defect and growth retardation (12, 19). Conditional inactivation of Csk in mouse T cells also leads to a pre-TCR/TCR-independent pathway of T-cell development as a result of hyperactivation of Lck and Fyn (23). Thus, Csk is the principal negative regulator of the Src family of PTKs and plays a critical role in mouse and T-cell development.

Unlike the Src family of PTKs, which are plasma membrane localized, Csk lacks a myristoylation sequence at its amino terminus and hence localizes primarily to the cytoplasm (18). In fact, the membrane-targeted form of Csk that contains the myristoylation sequence of Src more actively suppressed the function of the Src family of PTKs (5). Therefore, it is postulated that Csk requires interaction with some plasma membrane-associated proteins for its translocation from the cytosol to the plasma membrane, where it exerts its actions. Recently a transmembrane adaptor protein has been shown to fulfill this role and is termed Cbp for Csk-binding protein (16) or PAG for phosphoprotein associated with glycosphingolipid-enriched domains (1). Cbp was shown in cell transfection studies to be essential for the membrane localization of Csk (1, 16), and it could increase the latter's activity through both binding and conformational change mechanisms (27).

Similar to Csk, Cbp is ubiquitously expressed and is found in T cells. It localizes exclusively to glycosphingolipid-enriched membrane microdomains or lipid rafts (1, 16). Lipid rafts are enriched in signaling molecules, such as the Src family PTKs and G proteins, and are proposed to serve as signaling platforms to facilitate the propagation of signaling cascades from various membrane-bound receptors and in many different cell types (11). Structurally, Cbp has a long cytoplasmic tail containing multiple tyrosine-based motifs (9 in mouse and 10 in human). Among these, tyrosine 314 in mouse Cbp (which corresponds to Tyr317 in human Cbp) has been shown to be essential for binding Csk in transiently transfected COS cells (1, 16). Cbp also possesses a carboxyl-terminal VTRL motif that mediates its physical interaction with the PDZ domain of the cytoskeletal linker protein, EBP-50 (ezrin/radixin/moesin-binding phosphoprotein of 50 kDa) (2, 13), as well as a number of proline-rich domains that might mediate its interactions with other SH3-containing signaling molecules.

Cbp is constitutively phosphorylated in resting human α/β T cells, and the phosphorylated Cbp binds significant amounts of Csk (1). Upon TCR engagement, Cbp is rapidly dephosphorylated with the concomitant release of Csk and resulting in the activation of Lck and Fyn. When Cbp is transiently overexpressed in Jurkat T cells, it inhibits TCR-mediated activation of nuclear factor of activated T cells and the secretion of interleukin-2 (IL-2). In addition, $CD4^+$ T cells isolated from

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mice that overexpress Cbp were hypoproliferative and secreted a smaller amount of IL-2 upon TCR stimulation (8). Taken together, these findings suggest that Cbp plays a negative role in TCR signaling, most likely by recruiting a greater amount of Csk to lipid rafts and thereby inhibiting the activation of the Src family of PTKs.

Given that Cbp appears to be the major recruiter of Csk into lipid rafts, where it exerts its negative effect on the Src family of PTKs, and that Csk plays an important role in T-cell development (9, 23, 24), it is pertinent to assess whether Cbp is equally indispensable in the physiology and functions of T lymphocytes. In this report, we explore the physiological role of Cbp in T cells by analyzing Cbp-deficient mice.

MATERIALS AND METHODS

Generation of Cbp-deficient mice. The cDNA for Cbp was obtained by reverse transcription-PCR of RNA isolated from mouse spleen and used to probe a mouse 129 genomic DNA library. A phage clone containing the last exon of Cbp was obtained. Restriction enzyme digestion, Southern blotting, and DNA sequencing were used to map the genomic locus. A targeting vector was assembled, linearized by NotI digestion, and transfected into E14.1 embryonic stem (ES) cells. Targeted ES cells were selected by G418 resistance, and homologous recombinants were confirmed by Southern blotting using a 5′ external probe as indicated in Fig. 1. Seven homologous recombinants were obtained, and two of them were injected into C57BL/6 blastocysts to generate chimeric mice for germ line transmission of the inactivated allele. The mutant mice from these two different ES cell clones exhibited comparable phenotypes. All mice used in our experiments have a mixed 129 and C57BL/6 genetic background.

Flow cytometry analyses of $Cbp^{-/-}$ mice. Single-cell suspensions were obtained from thymus, spleen, lymph nodes, bone marrow, and peritoneal cavity of mice as described previously (29). Cells were stained with an optimal amount of fluorescein isothiocyanate-, phycoerythrin-, and biotin-conjugated antibodies to cell surface antigens. Biotin-conjugated antibody staining was revealed by a second step, streptavidin-CyChrome staining. The following antibodies, purchased from BD Pharmingen (San Diego, CA), were used in this study: anti-immunoglobulin M (IgM) (R6-60.2), anti-IgD (11-26C.2a), anti-B220 (RA3-6B2), anti-CD3ε (145-2C11), anti-CD4 (L3T4), anti-CD5 (53-7.3), anti-CD8 (53-6.7), anti-CD19 (1D3), anti-CD25 (7D4), anti-CD43 (S7), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-TCRβ (H57-597), anti-TCRVβ6 (RR4-7), and anti-TCRVβ8 (MR5-2). Data were collected on a FACScan (BD, Mountain View, CA) and analyzed using CellQuest software.

Intracellular calcium analysis. Thymocytes (5×10^6) were loaded with Indo-1 AM (2 μM; Molecular Probes) in the loading buffer (1 mM $CaCl_2$; 1 mM $MgCl_2$; 1% fetal calf serum in phosphate-buffered saline) for 45 min at 37°C. Cells were stained with phycoerythrin–anti-CD4, fluorescein isothiocyanate–anti-CD8, and biotinylated anti-TCRβ antibodies on ice for 15 min. After 30 min of incubation at room temperature, the cells were stimulated with streptavidin (5 μg/ml) to cross-link the TCRs. Calcium flux was monitored on an LSR II flow cytometer (Becton Dickinson) in real time for 15 min. $CD4^+$ $CD8^+$ double-positive cells and $CD4^+$ $CD8^-$ and $CD4^-$ $CD8^+$ single-positive cells were selectively analyzed for their abilities to flux calcium.

Measurement of T-cell proliferation and cytokine secretion. T cells were purified from lymph nodes of mice using mouse T-cell enrichment columns (R&D system). The purity of the negatively selected T cells was consistently >90% as assessed by flow cytometry using anti-CD3ε antibody. Stimulation of T cells was performed for 48 h at 37°C using 96-well plates that were precoated with various concentrations of monoclonal anti-mouse CD3ε antibodies in the absence and presence of soluble anti-CD28 antibody. To measure proliferation, cells were pulsed with 1.0 μCi [^{3}H]thymidine (Amersham) per well for the last 10 h of incubation. The amount of [^{3}H]thymidine incorporation was measured using a β-scintillation counter (Perkin-Elmer, Life Sciences). To measure cytokine secretion, cell culture supernatant was collected and assayed using the various cytokine enzyme-linked immunosorbent assay (ELISA) kits from BD Pharmingen. All assays were performed in triplicate, and the experiments were repeated at least five times.

Lipid raft purification. Thymocytes (4×10^8) were lysed in 0.05% Triton X-100 in TNEV buffer (150 mM NaCl, 5 mM EDTA, and 25 mM Tris-HCl, pH 7.4), followed by addition of an equal volume of 80% sucrose in lysis buffer and overlaid with 30% and 5% sucrose in the same buffer, respectively. The fractionation was performed in a SW60Ti rotor for 16 to 18 h at 4°C and at 200,000 × *g*. Eleven fractions were collected, and the lipid raft fractions (corresponding to third, fourth, and fifth fractions) were solubilized in 10 mM octyl glucoside (Sigma).

T-cell stimulation and immunoblotting. Purified lymph node T cells were incubated with 10 μg/ml biotinylated anti-TCRβ antibodies for 30 min at 4°C. Cells were washed and further stimulated with 5 μg/ml streptavidin at 37°C for various times. After stimulation, cells were lysed and analyzed by Western blotting antibodies against various signaling molecules: anti-Cbp (Exbio); anti-phospho-PLCγ1 and anti-phospho-LAT (Tyr191) (Cell Signaling Technology); and anti-phospho-ERK, anti-Csk, anti-ERK2, anti-PLCγ1, and anti-LAT (Santa Cruz). A rabbit polyclonal antibody directed against the N terminus of Cbp was raised using a peptide encompassing amino acids 42 to 56. The glycosphingolipid GM1 was detected with the horseradish peroxidase-conjugated cholera toxin B subunit (Sigma-Aldrich). Immunoblottings were visualized by enhanced chemiluminescence detection (Pierce, Rockford, IL)

Measurement of basal serum Ig, auto- and antigen-specific antibodies. To determine basal serum immunoglobulin levels, sera were obtained from 6- to 8-week-old mice, serially diluted, and added to ELISA plates that were precoated with anti-Igκ antibodies. The levels of serum Ig of various classes were assayed using commercially available kits (BD Pharmingen) according to the manufacturer's instructions. For the detection of autoantibodies, sera were taken from 9- to 18-month-old mice, and the presence of anti-double-stranded DNA (dsDNA) antibodies was measured using poly(dAdT) (Sigma) for coating and horseradish peroxidase-conjugated anti-IgG or biotinylated anti-IgM antibody and streptavidin-conjugated horseradish peroxidase for detection. To examine T-cell-dependent immune response, 10- to 12-week-old mice were immunized intraperitoneally with 100 or 5 μg of alum-precipitated NP_{17}-chicken globulin in a high- or low-dose regime, respectively. Sera were obtained 7, 14, and 28 days after challenge to detect NP-specific antibodies using ELISA. For secondary immune response, the mice were injected with 5 μg of the same antigen 45 days after primary challenge, and sera were collected 7 days later.

Peripheral T-cell tolerance to SEB. Five- to six-week-old mice were injected intraperitoneally with 100 μg of staphylococcal enterotoxin B (SEB) (Toxin Technology) on day 0. Peripheral blood was taken on days 0, 2, 5, 10, and 16 and analyzed by flow cytometry using antibodies against CD4, CD8, TCRβ8, and TCRβ6.

RESULTS

Generation of viable Cbp-deficient mice. Cbp is a transmembrane adaptor protein that recruits Csk to lipid rafts where the latter is postulated to exert its negative regulatory effect on the Src family of tyrosine kinases (1, 16). The inactivation of Csk in the mouse germ line has been shown to impair T-cell development and selection in vivo (9, 23, 24). To determine if Cbp has a similar role in T-cell physiology, we have inactivated the gene in mouse embryonic stem cells by deleting its last coding exon (from amino acid (aa) 311 onwards). This would abolish the critical Tyr-314 residue and the C-terminal VTRL motif, which are required for Cbp's interaction with Csk and EBP-50, respectively (2, 13) (Fig. 1A). Therefore, our targeting strategy would prevent Cbp from binding Csk and EBP-50 in the event that a truncated Cbp protein is ever produced.

The inactivation of the *cbp* gene in mice was verified by Southern blotting (Fig. 1B). Northern blot analysis further showed that there was no steady-state full-length or truncated *cbp* mRNA present in the mutant cells (Fig. 1C). To ascertain that we have established a null mutation of *cbp* in mice, we directly examined whether the Cbp protein was completely disrupted. To this end, we prepared whole-cell lysate from thymocytes of wild-type and mutant animals, separated them into lipid rafts and cytosolic fractions, and subsequently probed them with two different antibodies that recognized the C-terminal (aa 97 to 432) and the N-terminal (aa 42 to 56) regions of Cbp, respectively. As shown in Fig. 1D, both antibodies

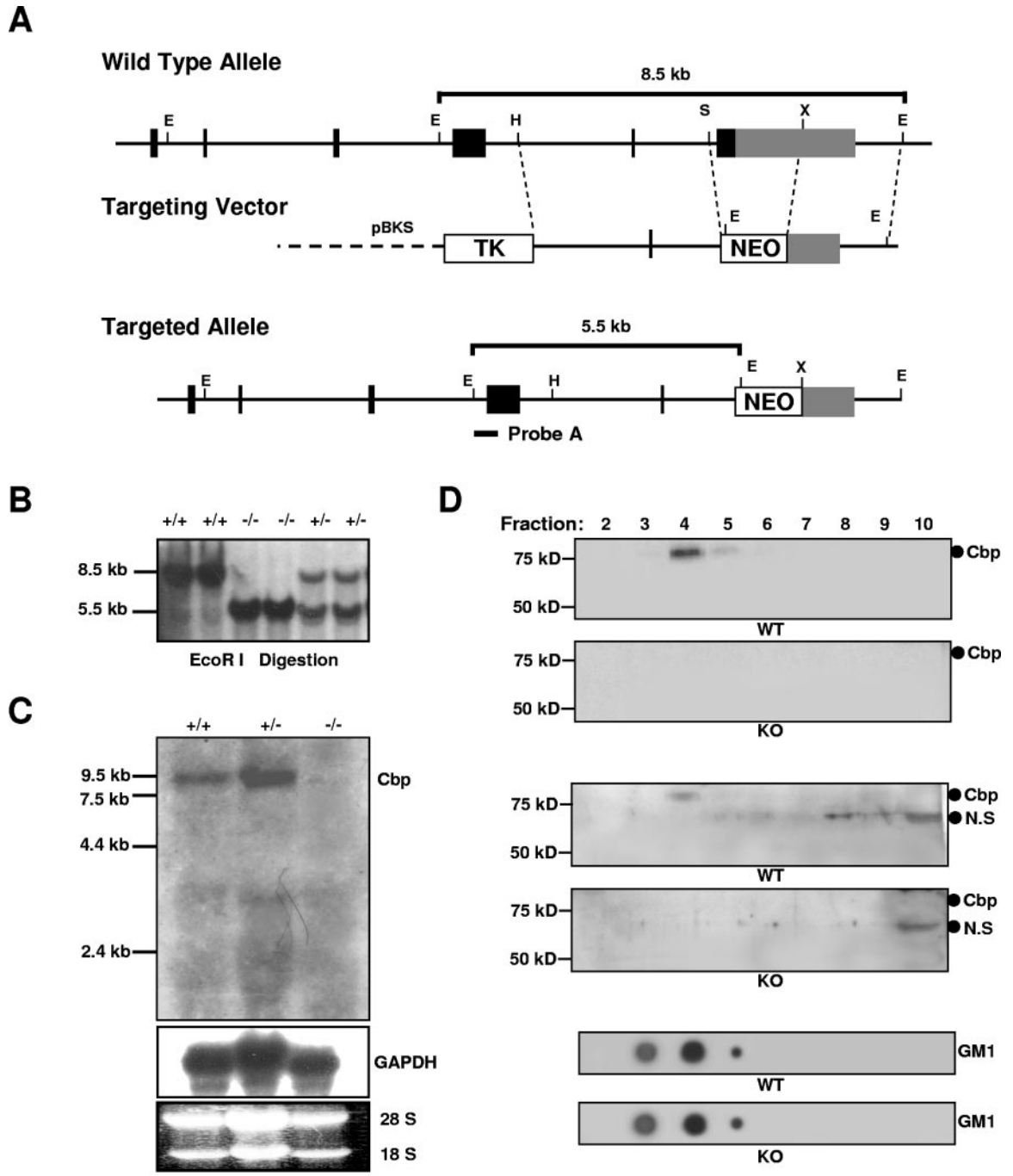

FIG. 1. Inactivation of the *cbp* gene locus. (A) Partial endonuclease restriction map of the wild-type *cbp* locus, the targeting construct, and the inactivated locus after homologous recombination. The restriction enzyme sites are as follows: B, BamHI; E, EcoRI; H, HindIII; X, XhoI; and S, SalI. The probe used to screen ES cells and mice, which distinguishes the 8.5-kb wild-type and 5.5-kb targeted alleles, is indicated. (B) Southern blot analysis of EcoRI-digested tail DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) Cbp knockout mice using the external probe. (C) Northern blot analysis of thymocyte RNA prepared from wild-type, heterozygous, and homozygous Cbp knockout mice using the full-length *Cbp* cDNA as a probe. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) blot is included as a control for loading and integrity of samples. (D) Western blot analysis of the Cbp protein in wild-type and Cbp knockout mice. Thymocytes were lysed in 0.05% Triton X-100 and subjected to sucrose density gradient centrifugation. Equal volumes of gradient fractions (fractions 2 to 10) were separated by SDS-PAGE and probed for the presence of Cbp using two different antibodies that recognized the C-terminal (panels 1 and 2) and N-terminal (panels 3 and 4) portions of Cbp. A dot blot of GM1 was included to indicate the gradient fractions that correspond to lipid raft fractions. A nonspecific (N.S) band present in both wild-type and mutant cell lysates using the antibody against the N terminus of Cbp is also included.

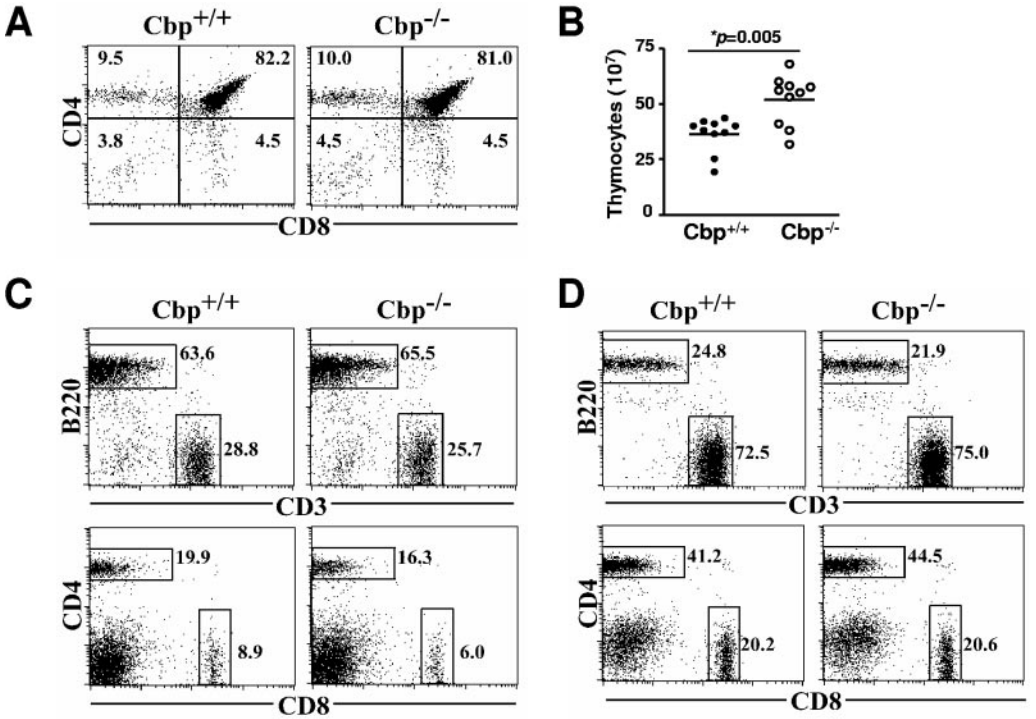

FIG. 2. Normal T-cell development in $Cbp^{-/-}$ mice. Flow cytometry analyses of T-cell populations in the thymus (A), spleen (C), and lymph nodes (D) of wild-type and mutant mice. Numbers indicate percentages of lymphocytes. Data shown are representative of more than five independent experiments. (B) Total number of thymocytes found in wild-type (black circles) and mutant (open circles) mice (4 to 5 weeks old). Each mouse analyzed was depicted as a single data point. Statistical significance was determined by a paired two-tailed Student *t* test.

could detect the 80-kDa Cbp protein in the lipid rafts (fractions 3, 4, and 5) but not in the cytosolic fractions of wild-type thymocytes. As expected, neither a full-length nor a truncated Cbp protein was detected in all cell lysate fractions obtained from mutant cells. This correlated well with the Northern blot analysis of mutant thymocytes, which indicated the absence of either a full-length or truncated *cbp* mRNA (Fig. 1C). Thus, Cbp has been inactivated in our mutant mice.

Interestingly, Cbp-deficient mice were viable and were born in the normal Mendelian ratio. They also survived to adulthood and beyond with no gross developmental defect. We have maintained Cbp mutant mice up to 20 months of age. This was in contrast to Csk-deficient mice, which suffered from early embryonic lethality (12, 19). Thus, although both Cbp and Csk are ubiquitously expressed and Csk-deficient mice are nonviable, Cbp is apparently not essential for mouse development.

Increased thymocyte population but normal T-cell development in Cbp-deficient mice. Since Csk has been shown to play an important role in T-cell differentiation, we focused our analyses on developing thymocytes and peripheral T cells in the mutant mice. T cells develop from CD4/CD8 double-negative to double-positive and finally to CD4 or CD8 single-positive cells in the thymus (25, 28). As shown in Fig. 2A, the absence of Cbp did not impair the maturation of thymocytes, since flow-cytometry analyses revealed that the different fractions of developing thymocytes were found in similar proportions in both wild-type and mutant mice. There was, however, a consistent and statistically significant overall increase in the total number of thymocytes found in 4- to 5-week-old $Cbp^{-/-}$ mice compared to age- and sex-matched littermate wild-type mice (Fig. 2B).

Peripheral T-cell populations in $Cbp^{-/-}$ mice also appeared normal, since equivalent fractions of $CD3^+$ and $CD4^+$ or $CD8^+$ cells were found in the spleen (Fig. 2C) and lymph nodes (Fig. 2D) of these mice compared to control mice. Analyses of older mice (9- to 12-month-old) also suggested that the populations of $CD62L^+$ $CD4^+$ $CD25^+$ regulatory T cells and $CD62L^{low}$ $CD4^+$ $CD44^{high}$ memory/effector T cells were indistinguishable between wild-type and mutant mice (unpublished data). B-cell development was also not affected in the bone marrow and peripheral lymphoid organs of $Cbp^{-/-}$ mice (unpublished data). Furthermore, enumeration of cells in the mutant mice also revealed that there was no overt increase in the number of B and T cells in the peripheral lymphoid tissues of these animals (Table 1). Taken together, these data indicate that Cbp deficiency does not affect T-cell development or homeostasis. This, again, is in contrast to the situation found in $Csk^{-/-}$ mice, which manifest a very early block in T-cell development (9).

Lipid raft-associated Csk is greatly reduced in the absence of Cbp. Given that significant amounts of Csk have been shown to associate with Cbp in lipid rafts (1, 16), we next examined if the amount of Csk in the lipid rafts of resting thymocytes could be altered in the absence of Cbp. Equal volumes of different gradient fractions (fractions 2 to 10) obtained from cell lysates of wild-type and Cbp-deficient thymocytes were blotted with antibodies against Cbp, Csk, and LAT. The presence of LAT denotes the lipid raft fractions of the cell lysates. As shown in

TABLE 1. B and T lymphocytes in spleen and lymph node[a]

Genotype (n = 10)	No. of cells (10^6) in:			
	Spl		MLN	
	$Cbp^{+/+}$	$Cbp^{-/-}$	$Cbp^{+/+}$	$Cbp^{-/-}$
IgM^+ $B220^+$	43.1 ± 4.6	42.8 ± 5.4	3.9 ± 0.5	4.4 ± 0.6
$CD3^+$ $CD19^-$	32.2 ± 3.5	33.5 ± 4.3	15.0 ± 2.0	18.4 ± 2.2
$CD4^+$ $CD8^-$	21.6 ± 2.8	22.8 ± 3.2	9.4 ± 1.4	11.7 ± 1.5
$CD4^-$ $CD8^+$	10.9 ± 1.1	10.6 ± 1.0	4.8 ± 0.5	5.8 ± 0.7

[a] Total B cells (IgM^+ $B220^+$), total T cells ($CD3^+$ $CD19^-$), CD4 single-positive T cells ($CD4^+$), and CD8 single-positive T cells ($CD8^+$) in spleen (Spl) and mesenteric lymph node (MLN) were enumerated. Data were shown as means ± SEM. Mice were age (4 to 5 weeks) and gender matched.

Fig. 3A (upper two panels), we were able to detect the presence of Csk in the lipid raft fractions (fraction 4 and to a lesser extent fraction 3) obtained from wild-type thymocytes. However, Csk was hardly detectable in the lipid rafts (fraction 4) of resting Cbp-deficient thymocytes. When equal protein amounts of lipid raft fractions (fraction 3, 4, and 5) were loaded to examine the presence of Csk (Fig. 3A, bottom two panels), the amount of Csk was significantly reduced in the lipid rafts fractions of mutant cells from that of wild-type cells, whereas the amounts of LAT in lipid rafts were comparable between wild-type and Cbp-deficient thymocytes (Fig. 3A, bottom panel). In addition, the amount of Csk in the whole-cell

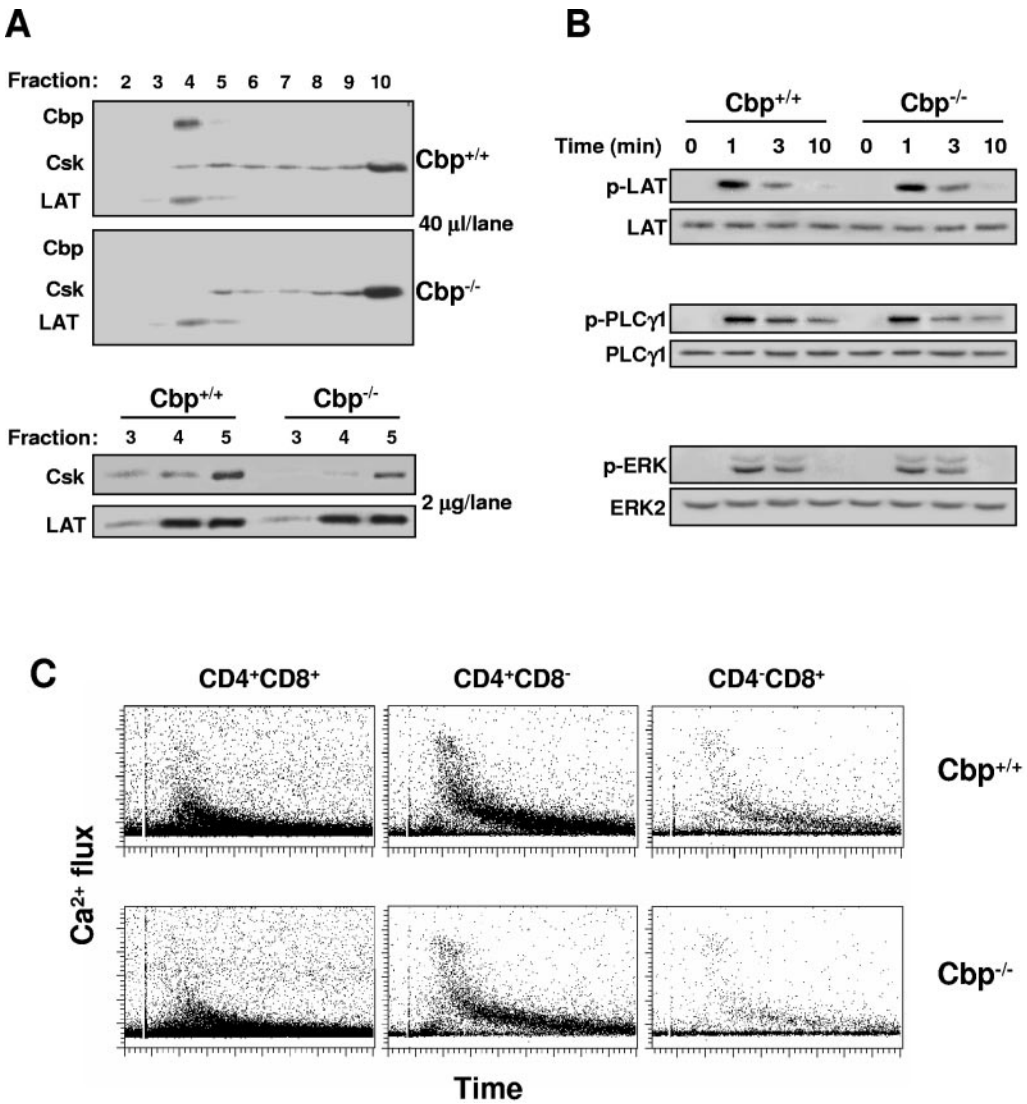

FIG. 3. Reduced Csk in lipid rafts but normal TCR-proximal signaling events in Cbp-deficient T cells. (A) The amount of Csk was reduced in the lipid rafts of Cbp-deficient thymocytes. Equal volumes (40 μl) of different gradient fractions (upper two panels) or equal protein amounts (2 μg/lane) of lipid raft fractions (bottom two panels) from thymocytes of wild-type and mutant mice were sequentially probed for the presence of Cbp, Csk, and LAT. (B) Downstream signaling events were intact following TCR activation in Cbp-deficient T cells. Mouse T cells were purified from lymph nodes of wild-type and mutant mice and stimulated for the indicated periods of time at 37°C with biotinylated anti-TCR antibody followed by cross-linking with streptavidin. The activation of LAT, PLCγ1, and ERK, as indicated by their phosphorylation status, was examined by Western blot analyses. (C) Intracellular calcium fluxes in response to TCR stimulation were comparable in wild-type and Cbp-deficient thymocytes. Thymocytes were preloaded with Indo-1 and subsequently stimulated with anti-TCR antibody. Intracellular $[Ca^{2+}]$ in $CD4^+$ $CD8^+$, $CD4^+$ $CD8^-$, and $CD4^-$ $CD8^+$ thymocytes is shown as a ratio of Indo-1 violet/blue fluorescence versus time.

lysate of thymocytes was not affected by the Cbp deficiency (unpublished data). Thus, Cbp is required to localize Csk to lipid rafts in primary resting thymocytes.

TCR-proximal signaling events are normal in absence of Cbp. Since lipid raft-associated Csk was greatly reduced in the absence of Cbp, we next determined if this would affect TCR-proximal signaling events. TCR engagement is known to lead to the phosphorylation of LAT and activation of both PLCγ1 and mitogen-activated protein kinases and to induce intracellular calcium flux (22). As shown in Fig. 3B, the activation of LAT, PLCγ1, and ERK1/2, as indicated by their phosphorylation status, appeared comparable between wild-type and Cbp-deficient T cells upon TCR ligation. We further examined TCR-induced calcium mobilization in both wild-type and Cbp-deficient thymocytes. Thymocytes were preloaded with the calcium indicator dye Indo-1 and were stimulated with biotinylated anti-TCR antibody followed by cross-linking with streptavidin. The intracellular calcium concentration was monitored over time using flow cytometry. As shown in Fig. 3C, TCR-induced calcium fluxes were comparable in either $CD4^+$ or $CD8^+$ and $CD4^+$ $CD8^+$ thymocytes obtained from wild-type and mutant mice. Similar results were also attained when a lower dose of streptavidin (2 μg/ml) was used to cross-link TCR (unpublished data). Hence, it appears that the absence of Cbp and the concomitant reduction of Csk in the lipid rafts of resting T cells do not affect the TCR-proximal signaling events.

In vitro T-cell responses are not compromised in the absence of Cbp. Since the initial localization of Csk in resting thymocytes was perturbed in the absence of Cbp but yet T-cell development and TCR-proximal signaling events remained normal in Cbp-deficient mice, we next asked if the absence of Cbp would affect T-cell responses rather than development. Since Csk negatively regulates the Src family of tyrosine kinases, which by themselves are critical for T-cell activation, it is conceivable that the absence of Cbp may lead to exaggerated T-cell responses.

To address this possibility, we examined the ability of mutant T cells to proliferate and secrete cytokines in response to TCR stimulation with and without CD28 costimulation. As shown in Fig. 4A, the engagement of the TCR via stimulation of the signal-transducing CD3 led to the proliferation of wild-type T cells in a dose-dependent manner. This proliferative response was further enhanced by the coengagement of the CD28 molecule. Interestingly, the absence of Cbp neither incapacitated nor exaggerated the proliferative responses of the mutant T cells. Likewise, the ability of T cells to produce cytokine after activation, as shown by the secretion of IL-2 and -4 and gamma interferon (Fig. 4B), was also indistinguishable between wild-type and mutant T cells. Taken together, the data suggest that the absence of Cbp does not affect T-cell responses in vitro.

Cbp-deficient mice have normal humoral immune responses. Since Csk, which interacts with Cbp, regulates the activation of the Src family of tyrosine kinases, and the impairment of Lyn, a member of this family, is known to lead to autoimmunity with high serum antibody titers in mice (10), we next assessed if B-cell responses in $Cbp^{-/-}$ mice would be altered. Examination of the serum antibody levels in Cbp-deficient mice revealed that there were no significant differences in the amounts of immunoglobulins of different classes in

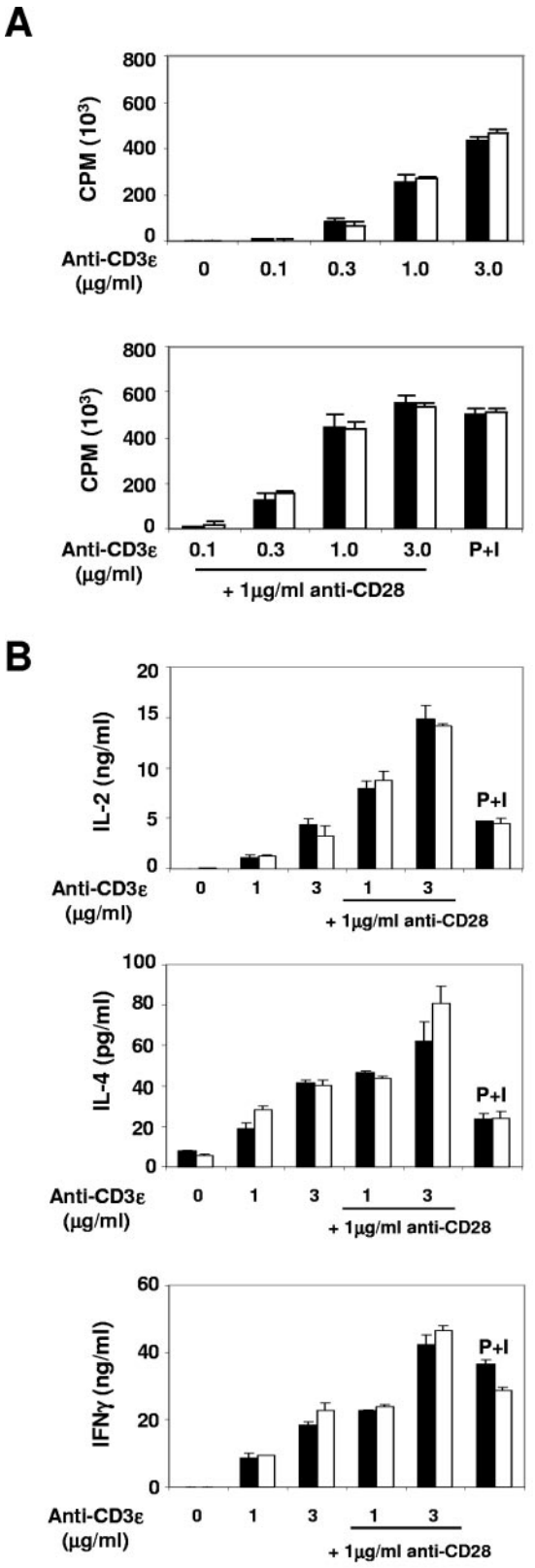

FIG. 4. T-cell proliferation and cytokine production in the absence of Cbp. Wild-type and mutant T cells were stimulated with various doses of anti-CD3 and anti-CD28 antibodies and examined for their (A) proliferation and (B) secretion of IL-2, IL-4, and IFN-γ. Data shown are representative of at least five independent experiments.

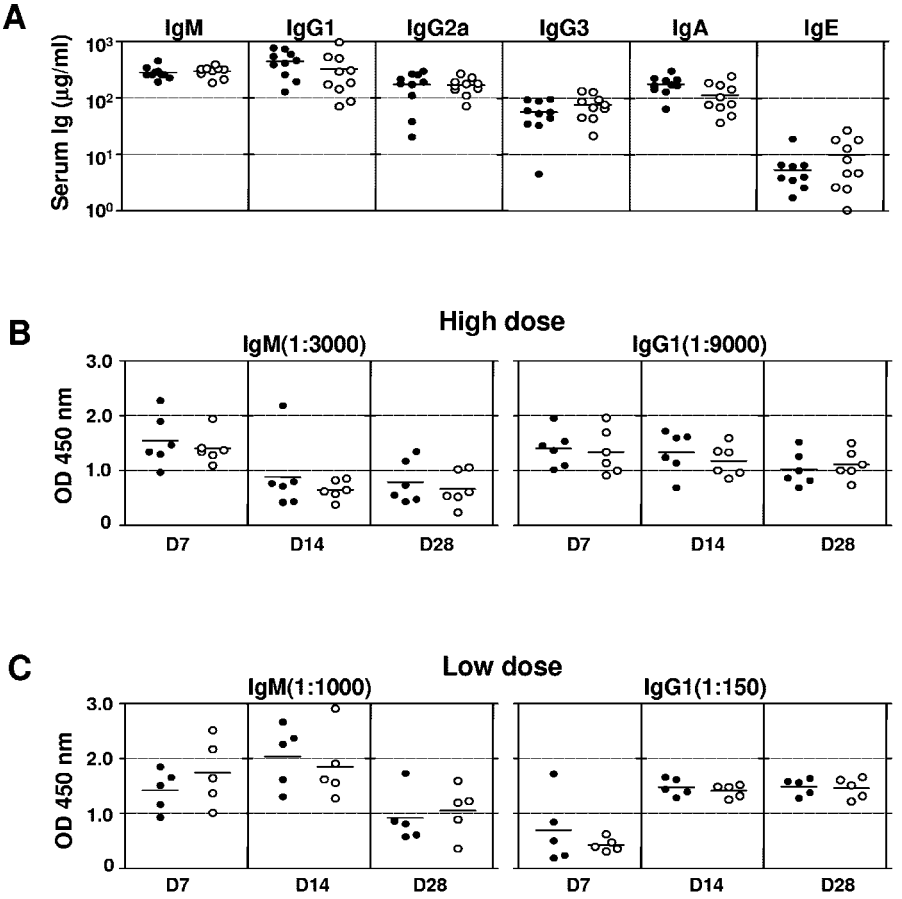

FIG. 5. T-cell-dependent humoral immune responses in $Cbp^{-/-}$ mice. Basal serum immunoglobulin levels of various classes (A) and primary T-cell-dependent humoral immune responses to a high dose (B) and low dose (C) of antigen were shown for wild-type (black circles) and $Cbp^{-/-}$ (open circles) mice at various time points after challenge (D, day). Each individual mouse is represented as a single data point.

the sera of these mice compared to those of control animals (Fig. 5A).

Antigens that elicit a specific antibody response from B cells can be classified as either T cell independent or T cell dependent according to their dependency on $CD4^+$ T-cell help. To determine if $Cbp^{-/-}$ mice could mount efficient immune responses against exogenous antigens, we first immunized the mice with a T-cell-independent antigen, NP-Ficoll. The antibody response to NP-Ficoll was mainly of the IgM and IgG3 class; again both wild-type and $Cbp^{-/-}$ mice responded equally well to NP-Ficoll challenge (unpublished data).

For the T-cell-dependent immune response, we first challenged the mice with either a high or low dose of the antigen NP-CG and assessed the magnitude of both the primary and secondary immune responses. The antibody response to NP-CG was mostly of the IgM and IgG1 classes. Again, as shown in Fig. 5B and C, the primary immune responses of $Cbp^{-/-}$ mice to either a high or low dose of T-cell-dependent antigen were indistinguishable from that of wild-type mice. Similar results were obtained when the mice were rechallenged with the same antigen in a secondary immunization regime (unpublished data). Thus, the lack of Cbp does not alter the ability of T cells to provide help to B cells in the T-cell-dependent humoral immune response.

Absence of Cbp did not affect peripheral B- and T-cell tolerance. To further determine if Cbp plays a role in T-cell activation in vivo, we examined the induction of peripheral T-cell tolerance after SEB challenge in mice. SEB is a superantigen that stimulates the proliferation of a subset of T cells whose TCR contains either a Vβ7, -8, or -17 chain (15). Administration of SEB in adult wild-type mice will result in the initial proliferation of these T cells at early time points, followed by their anergy and deletion at later time points. As shown in Fig. 6A, there were an initial increase and a subsequent decrease in the populations of $CD4^+$ $V\beta8^+$ and $CD8^+$ $V\beta8^+$ T cells following the administration of SEB in wild-type mice. Examination of the $CD4^+$ $V\beta8^+$ and $CD8^+$ $V\beta8^+$ T-cell populations in $Cbp^{-/-}$ mice revealed that they were indistinguishable from that of the wild-type T cells. As a control, the populations of $CD4^+$ $V\beta6^+$ and $CD8^+$ $V\beta6^+$ T cells, which were not stimulated by SEB, remained fairly constant for the duration of the challenge in both wild-type and Cbp-deficient mice. Taken together, the data indicate that Cbp does not play a role in the induction of T-cell peripheral tolerance to SEB.

Finally, we assess if Cbp-deficient mice have a propensity to develop autoimmunity by assaying for pathological autoantibodies against dsDNA in the sera of aged $Cbp^{-/-}$ mice. As shown in Fig. 6B, IgG autoantibodies were not detected in 9-

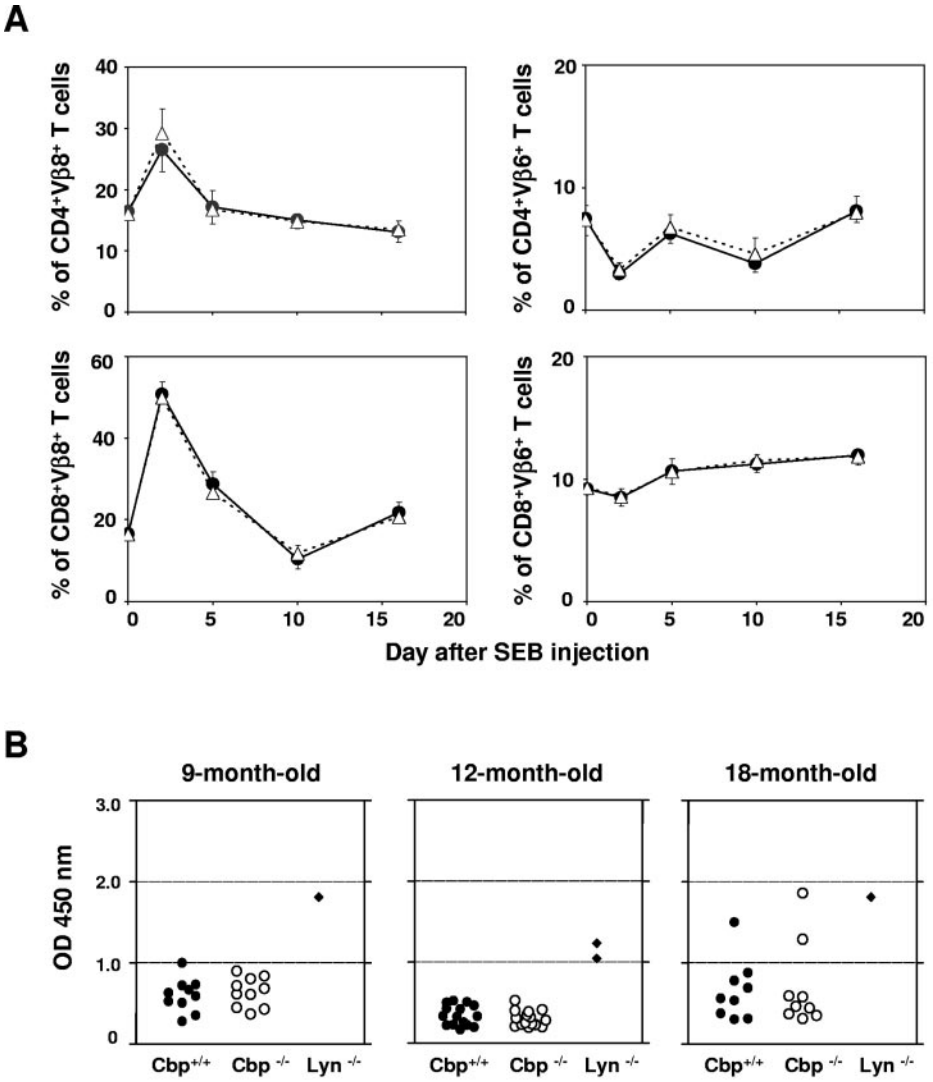

FIG. 6. Unaffected B- and T-cell tolerance in the absence of Cbp. (A) Wild-type (black circles) and $Cbp^{-/-}$ (open triangles) mice were given SEB at day 0, and the fractions of responding $CD4^+$ $V\beta8^+$ and $CD8^+$ $V\beta8^+$ and control $CD4^+$ $V\beta6^+$ and $CD8^+$ $V\beta6^+$ cells were quantified at various days after antigenic challenge. (B) Serum levels of IgG auto-antibodies against dsDNA were quantified in aged wild-type (black circles), $Cbp^{-/-}$ (open circles), and control $Lyn^{-/-}$ (black diamonds) mice. Each data point represents an individual mouse analyzed.

to 12-month-old $Cbp^{-/-}$ mice, unlike the situation in autoimmune-prone $Lyn^{-/-}$ mice, which developed autoantibodies by 6 months of age (10). IgG autoantibodies to dsDNA were found in 18-month-old $Cbp^{-/-}$ mice, but the occurrence was rare and did not differ significantly from that of $Cbp^{+/+}$ mice. The similar results were also obtained for IgM autoantibodies (unpublished data). Hence, the absence of Cbp does not lead to the preferential development of autoimmunity in mice.

DISCUSSION

We report here the generation and characterization of mice deficient for the lipid raft-associated transmembrane adaptor protein Cbp, whose primary role is to recruit the cytoplasmic PTK Csk to lipid rafts, where the latter inactivates the Src family of PTKs (1, 16). The importance of Csk in early embryonic development was evidenced by the growth retardation and necrosis of neural tissues in Csk-deficient mice, which resulted in their failure to progress beyond day 10 of embryonic development (12, 19). Interestingly, as we have shown here, Cbp-deficient mice were viable and did not show any gross defect in development. This suggests that Cbp and its association with Csk are not critical for embryogenesis.

Both Csk and Cbp are expressed in T cells (1, 16, 19). Csk had been shown to be essential for early T-cell development, since its impairment led to the arrest of T-cell differentiation (9). Conditional inactivation of Csk in T cells also resulted in an abnormal $CD4^+$ T-cell population in the periphery (23, 24). Since Cbp was shown to be the main adaptor that recruited Csk to lipid rafts (1, 16), it was therefore a surprise that T-cell development in the thymus or peripheral organs was not grossly altered in our Cbp mutant mice. This result was also consistent with a previous report that showed normal T-cell development in transgenic mice overexpressing a dominant-

negative form of Cbp (8). Thus, the available data indicate that Cbp is not essential for T-cell differentiation.

Cbp has also been shown in cell-based studies to play an inhibitory role in T-cell activation, since its overexpression in Jurkat T cells inhibited TCR-induced activation of nuclear factor of activated T cells (1). Moreover, murine T cells that overexpressed wild-type Cbp were hypoproliferative and secreted reduced amounts of IL-2 after TCR stimulation, whereas those that overexpressed a dominant-negative form of Cbp, in which tyrosine 314 was mutated into phenylalanine and therefore unable to bind Csk, were hyperresponsive (8). These findings suggest that Cbp is involved in the negative regulation of TCR signaling, most likely by recruiting Csk. It was therefore a surprise that T-cell activation was not grossly altered in the absence of Cbp. Activation of Cbp-deficient T cells via the TCR and the costimulatory CD28 molecule did not lead to excessive or reduced cell proliferation and cytokine secretion. In line with the normal in vitro responses of Cbp-deficient T cells, the TCR-proximal signaling events including the phosphorylation of key signaling molecules, such as LAT, PLCγ1, and ERK, and intracellular calcium flux were intact in Cbp-deficient T cells. These data contrasted with those obtained using transgenic mice that overexpressed Cbp, in which the T cells were shown to be hypoproliferative, secreted less IL-2, and had slightly reduced LAT phosphorylation and calcium flux in response to TCR stimulation (8). A likely explanation is that in this case, the overexpression of Cbp may mop up other as yet unidentified proteins that interact with Cbp, some of which may be positive or negative regulators of TCR signaling, therefore leading to changes in T-cell activation. This is conceivable, since Cbp possesses nine tyrosine residues which potentially can bind other signaling molecules and only Tyr 314 has been shown to bind Csk. Future experiments to identify other Cbp-interacting proteins may help to address this possibility.

Although the lack of Cbp did not overtly affect T-cell activation, our biochemical analyses of Cbp-deficient thymocytes did yield some insights into the mechanisms of Csk localization in lipid rafts. It has been hypothesized that Csk is recruited to lipid rafts primarily via the interaction of its SH2 domain with Y314 of Cbp (1, 16). Consistent with this, we were not able to detect a substantial amount of Csk in lipid rafts isolated from Cbp-deficient thymocytes compared to that for wild-type cells. This would suggest that the lipid raft localization of the majority of Csk is mediated by Cbp. But this does not exclude the existence of Cbp-independent mechanisms for the recruitment of Csk into lipid rafts, since we did detect minute amount of Csk in the lipid rafts of Cbp-deficient thymocytes. Along this line of argument, it has been reported that the SH2 domain of Csk could bind other molecules, such as Dok-3 (17), SIT (20), LIME (3), paxillin, and focal adhesion kinase (21), a number of which can translocate to the lipid rafts after T-cell activation. However, these molecules may not be as efficient as Cbp in localizing Csk in lipid rafts. Despite this, it appears that the much-reduced amount of lipid raft-associated Csk in Cbp-deficient T cells is sufficient to maintain normal T-cell differentiation and activation.

Finally, it was speculated that the lack of Cbp/Csk complex in the lipid rafts of resting T cells would cause partial activation of the Src PTKs, leading to a lowered signaling threshold for T-cell activation (11). Such "constitutively preactivated" T cells might make the mutant mice more susceptible to the development of autoimmune diseases or lymphomas. However, the Cbp-mutant mice did not succumb to any of these conditions when the mice were kept up to 18 months of age in our specific-pathogen-free facility. This result indicates that Cbp is probably not essential in the maintenance of T-cell homeostasis in vivo.

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