T Cell Responses in Calcineurin $\alpha$–deficient Mice

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Summary

We have created embryonic stem (ES) cells and mice lacking the predominant isoform ($\alpha$) of the calcineurin $\alpha$ subunit (CNA$\alpha$) to study the role of this serine/threonine phosphatase in the immune system. T and B cell maturation appeared to be normal in CNA$\alpha$-/- mice. CNA$\alpha$-/- T cells responded normally to mitogenic stimulation (i.e., PMA plus ionomycin, concanavalin A, and anti-CD3e antibody). However, CNA$\alpha$-/- mice generated defective antigen-specific T cell responses in vivo. Mice produced from CNA$\alpha$-/- ES cells injected into RAG-2-deficient blastocysts had a similar defective T cell response, indicating that CNA$\alpha$ is required for T cell function per se, rather than for an activity of other cell types involved in the immune response. CNA$\alpha$-/- T cells remained sensitive to both cyclosporin A and FK506, suggesting that CNA$\beta$ or another CNA-like molecule can mediate the action of these immunosuppressive drugs. CNA$\alpha$-/- mice provide an animal model for dissecting the physiologic functions of calcineurin as well as the effects of FK506 and CsA.

Calcineurin, also known as protein phosphatase 2B, is a calcium- and calmodulin-dependent serine/threonine protein phosphatase (1, 2). It is expressed in all mammalian tissues examined and is most abundant in the brain (2). In lymphocytes, calcineurin is the major soluble calmodulin-binding protein (3). Calcineurin is a heterodimer consisting of a catalytic subunit (CNA$\alpha$; 61 kD) and a regulatory subunit (CNB; 19 kD). The phosphatase activity of the $\alpha$ subunit is regulated by calcium through both calmodulin and the B subunit (4). There are two genes encoding closely related (~80% identical) CNA subunit isoforms, CNA$\alpha$ and CNA$\beta$, in the mouse, human, and rat genomes (5–8 and our unpublished data). The $\alpha$ isofrom is the predominant isofrom found in brain, thymus, and T cells (1, 9, 10, and our unpublished data). Even though CNA$\alpha$ and CNA$\beta$ have similar functions in vitro, the physiologic functions of the different calcineurin $\alpha$ isoforms are not yet defined.

CsA and FK506 are immunosuppressive drugs used to prevent graft rejection after organ transplantation. In vitro studies from several groups have suggested that CsA and FK506 exert their immunosuppressive effects by inhibiting calcineurin’s phosphatase activity (11–15). Complexes formed by CsA and FK506 and their respective binding proteins inhibit calcineurin’s phosphatase activity (16–18). This loss of phosphatase activity correlates with inhibition of T cell activation (19).

To distinguish the roles of CNA$\alpha$ and CNA$\beta$ in T cell activation, we generated embryonic stem (ES) cells and mice lacking a functional CNA$\alpha$ gene. The composition and distribution of T cell subsets were normal in CNA$\alpha$-/- mice. CNA$\alpha$-/- and wild-type T cells displayed similar responses to mitogenic stimulation. Surprisingly, CNA$\alpha$-/- mice gave deficient in vivo antigen-specific T cell responses. The residual CNA$\beta$ activity in CNA$\alpha$-/- T cells cannot substitute for CNA$\alpha$ activity in the immune response. CNA$\alpha$-/- T cells remained sensitive to CsA and
FK506, suggesting that another molecule, probably CNAβ, can mediate the action of CsA and FK506 in T cells.

Materials and Methods

Construction of Targeting Vector. The targeting construct used to disrupt the CNAα gene was designed for implementing the double-selection technique previously described (20, 21). Murine CNAα cDNA was cloned by PCR amplification (22) from mouse brain total cDNA using primers corresponding to CNAα cDNA sequence (reference 8; GenBank accession No. J05479). The murine CNAα cDNA was used to screen a 129/SvJ liver genomic library (Stratagene, La Jolla, CA). A bacteriophage clone encoding part of the CNAα catalytic domain (8) was isolated and designated MCAL-1. A 13-kb EcoRI fragment from clone MCAL-1 was subcloned into Bluescript containing the thymidine kinase gene (tk) (21). The intron-exon boundaries of this genomic fragment were defined by restriction enzyme site mapping and nucleotide sequence analysis (22). The neomycin phosphotransferase gene (neo) was inserted into an MluI site in the exon encoding nucleotides 572–717 of the mouse CNAα mRNA sequence (8). Both neo and tk were under the control of the phosphorcytate kinase promoter.

Transfection and Selection of Mutant ES Cells. J1 ES cells were grown on feeder layers of γ-irradiated embryonic fibroblast (EF) cells as described (23). 1.5–2 × 10⁶ J1 cells at passage 9–10 were trypsinized and resuspended in 1 ml of electroporation buffer (24) that had been modified to contain 137 mM NaCl. 50 μg of construct DNA was introduced into J1 ES cells by electroporation. The ES cells were then grown in G418 and 1–[2-deoxy-2-fluorobeta-D arabinofuranosyl]-5-iodouracil (FIAU) as described previously (23). Surviving clones were picked 8–10 d after selection, and DNA was extracted for Southern blot analysis.

Generation of Germline Chimeras. Heterozygous ES cells were injected into C57BL/6J blastocysts and reimplanted into the uteri of Black Swiss pseudo pregnant female mice as described (25). Agouti male offspring (derived from the 129 ES cells) were mated to Black Swiss or C57BL/6J females. Germine transmission of the mutated allele of the CNAα gene was monitored by Southern blot analysis of tail DNA from the agouti F1 offspring (see Fig. 1). Homozygous CNAα−/− mice were obtained by backcrossing heterozygous knockout ES cells from a single clone were plated onto each 10-cm plate on G418-resistant medium, with 1 μg/ml OVA. Supernatants from 2 × 10⁶ T cell-depleted and irradiated C57BL/6 spleen cells (as sources of exogenous APCs), or with OVA plus 10 U/ml IL-2. 60 h after stimulation, 1 μCi [3H]thymidine was added per well, and incorporated radioactivity was assayed 6 h later.

T cell–derived cytokines were measured using standard procedures (30). Lymph node cells from immunized mice were harvested and cultured with 1 mg/ml OVA. Supernatants from 2 × 10⁶ cells/200 μl per well in 96-well plate were harvested after 24 h. The IL-2 activity in the supernatants was measured by proliferation of HT-2 cells in the presence of αβ-4 antibody (11B11). At 24 h, the HT-2 culture was pulsed with 1 μCi/well of [3H]thymidine for 6 h. Supernatants from 6 × 10⁵ cells/200 μl per 96-well plate were harvested after 60 h. The amounts of IFN-γ in the supernatants were measured by ELISA.

To assay polyclonal T cell responses, spleen and lymph node cells from wild-type and CNAα−/− mice of Black Swiss/129 background were harvested and cultured in duplicates at 2 × 10⁶ cells/well with 2.5 mg/ml PMA plus 75 μg/ml ionomycin, or 2.5 μg/ml ConA, or soluble αCD3ε antibody (1:500 dilution of 145.2c11 hybridoma supernatant) (cCD3), or plate-bound αCD3ε antibody (plate coated with 10 μg/ml of purified αCD3ε antibody) (x-CD3). After 60 h of stimulation, cultures were pulsed with 1 μCi/well [3H]thymidine for 6 h.

Assessment of Calcineurin in T Cells. Spleen and lymph node cells harvested from wild-type and CNAα−/− mice were dispersed into single-cell suspensions. RBC were lysed by the addition of 1–5 ml of Tris/NaCl solution for 5 min at room temperature. Cell suspensions were filtered with a nylon mesh and washed twice with staining medium (HBSS with reduced phenol red, sodium azide, BSA, and EDTA). 0.5 × 10⁶ cells were resuspended in 25 μl staining medium and incubated for 15 min on ice with 1 μg of PE- or FITC-labeled antibodies (Pharmingen, San Diego, CA) in 10 μl staining medium. Cells were washed once and fixed with 0.5% formamide in staining medium. Flow cytometry was carried out using a flow cytometer (Cytofluorograf II. Becton-Dickinson, San Jose, CA). Each analysis recorded 20,000 cells.

H-2 Genotyping. The presence of H-2k alleles in the CNAα−/− mice of Black Swiss/129 background was ascertained by PCR amplification of an I-Eαb fragment (28) using the following primers: Eα5 (AGTCTTCCCAGCCTTCACACTCAGAGGTAC) and Eα3′ (CATAGCCCCAAAATGTCTGT ACCTCTGAGGAG) (28a).

In Vivo Immunization and In Vitro T Cell Proliferation and Cytokine Production Assays. 8–10-wk-old wild-type and CNAα−/− mice of Black Swiss/129 background were immunized with 300 μg of trinitrophenol (TNP)-conjugated OVA in CFA via foot pad injection (29). 10 d after immunization, lymph node cells were harvested. 2 × 10⁶ lymph node cells per well were cultured in triplicates in 96-well plates containing OVA alone, OVA plus 2 × 10⁶ T cell-depleted and irradiated C57BL/6 spleen cells (as sources of exogenous APCs), or with OVA plus 10 U/ml IL-2. 60 h after stimulation, 1 μCi [3H]thymidine was added per well, and incorporated radioactivity was assayed 6 h later.

RAG-2−/− mice, and tissues were dissociated into single-cell suspensions. RBC were lysed by the addition of 1–5 ml of Tris/NaCl solution for 5 min at room temperature. Cell suspensions were filtered with a nylon mesh and washed twice with staining medium (HBSS with reduced phenol red, sodium azide, BSA, and EDTA). 0.5 × 10⁶ cells were resuspended in 25 μl staining medium and incubated for 15 min on ice with 1 μg of PE- or FITC-labeled antibodies (Pharmingen, San Diego, CA) in 10 μl staining medium. Cells were washed once and fixed with 0.5% formamide in staining medium. Flow cytometry was carried out using a flow cytometer (Cytofluorograf II. Becton-Dickinson, San Jose, CA). Each analysis recorded 20,000 cells.

145.2c11 hybridoma supernatant) (cCD3), or plate-bound αCD3ε antibody (plate coated with 10 μg/ml of purified αCD3ε antibody) (x-CD3). After 60 h of stimulation, cultures were pulsed with 1 μCi/well [3H]thymidine for 6 h.

Assessment of Calcineurin in T Cells. Spleen and lymph node cells harvested from wild-type and CNAα−/− mice were dispersed into single-cell suspensions. RBC were removed by lysis (see above). The cell concentrations were adjusted to 1.5–2.0 × 10⁶ in 2 ml of PBS containing 5% FCS and 4 mM EDTA. The cell suspensions were enriched for T cells using Mouse T Cell Enrichment Columns (R&D Systems, Inc., Minneapolis, MN). The T cells were lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 15% glycerol, 0.1 mM EGTA, 1 mM EDTA, 0.5 mM DTT, 50 μg/ml PMSF, 50 μg/ml soybean trypsin inhibitor, 5 μg/ml aprotinin, and 5 μg/ml leupeptin. Aliquots of 8 × 10⁶ cells/50 μl of lysis buffer were frozen until further analysis.

The amount of calcineurin Aα in T cell extracts was assessed by Western blots (31). 20 μg of T cell extract were fractionated by SDS-PAGE on a 16% Tris/glycine gel (Novex, San Diego,
CA) at 150 V (constant voltage) and transferred to a polyvinylidene
fluoride membrane (Immobilon) at 100 V for 1.5 h. After trans-
fer, the membrane was blocked overnight in M-Blotto at 4°C.
The membranes were washed and HPR-conjugated donkey anti-rabbit
secondary antibody diluted 1:10,000 (Amersham Corp., Arling-
ton Heights, IL). The membranes were washed in Tris-buffered
saline Tween (TBST) and developed with the enhanced chemilu-
minescence Western blotting detection system (Amersham).

Calcineurin Phosphatase Assay. The phosphatase assay was as
described in Martin and Wiederrecht (32).

Results

Generation of CNAα−/− Mice. Calcineurin Aα knock-
out (CNAα−/−) mice were produced by standard methods
(25) after homologous recombination in ES cells. The gene
 targeting vector was constructed by inserting the neo gene
into an exon that encodes part of the calcineurin catalytic
domain and by inserting the tk gene outside the region of
homology (Fig. 1 a). Linearized construct DNA was trans-
ferred into J1 ES cells and colonies were selected for neo-
mycin and FIAU resistance (21, 23). DNA from individual
clones was analyzed by Southern blot analysis after diges-
tion with MscI and hybridization with the 1.2-kb probe
(Fig. 1). A novel 7.5-kb fragment, as well as the 18-kb
fragment found in wild-type ES cell DNA, were found in
CNAα−/− ES cell DNA (Fig. 1 b; data not shown).

Heterozygous CNAα+/− ES cells were then injected into
C57BL/6 blastocysts and some of the resultant chimeric mice passed the mutated gene onto the next generation
when mated with Black Swiss or C57BL/6 mice. CNAα−/− mice were obtained by mating the heterozygous
CNAα−/− mice.

Generation of CNAα−/−/RAG-2−/− Chimeras. CNAα−/−
ES cells were grown in 1.5 mg/ml G418 to select ES cells
lacking both copies of functional CNAα genes (21). DNA
was extracted from surviving clones and analyzed by
Southern analysis. CNAα−/− clones were identified by
their lack of the endogenous 18-kb MscI fragment (Fig. 1 b
and data not shown). The functional disruption of the
CNAα gene was confirmed by lack of CNAα expression
in CNAα−/− ES cells. RNA from wild-type, CNAα+/−,
and CNAα−/− ES cells were characterized by Northern
analysis using the 5' end of CNAα cDNA (reference
8; nucleotides 95–714) as probe. CNAα mRNA was de-
tected in wild-type ES cells and in CNAα+/− ES cells, but
not in CNAα−/− ES cells (Fig. 1 c).

CNAα−/− ES cells were injected into RAG-2−/− blasto-
cysts of either B6/129 or FvB background to generate so-
matic chimeras (27). In RAG-2−/− mice, there are no ma-
ture T and B cells because of the inability of their Ig or
TCR genes to undergo rearrangement (26). Therefore, all the
mature T and B cells in the CNAα−/−/RAG-2−/− chimera-
s should be derived from the injected CNAα−/− ES cells.

Figure 1. The CNAα gene was targeted by homologous re-
combination, and cells homozy-
gous for this mutation did not
produce CNAα mRNA or polypeptide. (a) Partial genomic
structure of the CNAα gene
(top), the targeting construct for
homologous recombination
(middle), and the targeted allele
(bottom). The neo gene was inserted
into an exon encoding nucle-
otides 572–717 of the mouse
CNAα mRNA sequence (8).
The 1.2-kb EcoR1 fragment was
used as a hybridization probe for
Southern analyses to identify ho-
omologous recombination events
(see b). The location of several
restriction enzyme sites (E, EcoR1; M, MscI; Mf, MluI) are
indicated. *Enzyme site in the
vector. (b) Southern analyses of
J1 ES cell DNA (lane 1), CNAα−/−
(lane 2), and CNAα+/− (lane 3) ES cells using the 5' end of calcineurin
α cDNA (nucleotides 95–714) as the probe. (d) Western analyses of T cell extracts from wild-type (lane 1) and mutant mice (lane 2) using CNAα-spe-
cific antibodies.
Calcineurin Activity in CNA\alpha<sup>-/-</sup> T Cells. The amounts of calcineurin in T cell extracts derived from CNA\alpha<sup>-/-</sup> and wild-type mice were compared by Western blotting and enzyme activity assays. A CNA\alpha-specific antibody only detected CNA\alpha polypeptide in wild-type T cell extracts, but not in CNA\alpha<sup>-/-</sup> T cell extracts (Fig. 1 d). A CNA\beta-specific antibody did not detect CNA\beta peptide in either wild-type or mutant T cells, even though this peptide could be readily detected in brain (our unpublished results).

Calcineurin activity (okadaic acid resistant and EGTA-sensitive phosphatase activity) was measured in the same wild-type and CNA\alpha<sup>-/-</sup> T cell extracts (32). The phosphatase activity in CNA\alpha<sup>-/-</sup> T cell extracts was 34% of the activity found in wild-type T cell extracts (169 ± 32 vs 500 ± 77 pmol substrate/min per mg protein). The residual calcineurin-like activity in the mutant T cells could be contributed by other calcineurin isoforms or by other related phosphatases. The phosphatase activity in both the wild-type and CNA\alpha<sup>-/-</sup> T cells was 90–95% inhibited by FK506. Our data, consistent with previous findings, suggested that \alpha is the predominant isoform in T cells. In the absence of CNA\alpha, CNA\beta does not seem to be increased.

Normal Development of T and B Lineage Cells. The composition and distribution of T and B cell lineage cells were normal in the thymus, spleen, lymph nodes, and bone marrow of CNA\alpha<sup>-/-</sup> mice based on staining with antibodies to TCR\alpha, TCR\beta, CD3, CD4, CD8, MHC class I and II, Thy-1, IL-2R\alpha, B220, IgM, IgG, IgD, IgA, IgE, CD23, S7, and CD5 (Fig. 2; data not shown). CNA\alpha<sup>-/-</sup> mice had populations of double-negative (CD4<sup>-</sup>CD8<sup>-</sup>), double-positive (CD4<sup>+</sup>CD8<sup>+</sup>), and single-positive (CD4<sup>-</sup>CD8<sup>+</sup> or CD4<sup>+</sup>CD8<sup>-</sup>) thymocytes that were comparable to wild-type litter mates (Fig. 2; data not shown). Staining with different VB antibodies (VB5, VB6, VB8, VB9, VB11, VB13, and VB14) showed that VB usage in the CNA\alpha<sup>-/-</sup> thymus was similar to that in the wild-type thymus (data not shown). These findings indicated that a functional CNA\alpha gene is not required for the maturation of either T or B lymphocytes.

Defective T Cell Responses to Protein Antigens. To determine if CNA\alpha is required for a normal immune response, we measured the responses of wild-type and CNA\alpha<sup>-/-</sup> mice to immunization with hapten–protein antigens. Wild-type and CNA\alpha<sup>-/-</sup> mice with at least one H-2<sup>b</sup> allele were

![Figure 2. Normal development of T and B lineage cells in CNA\alpha<sup>-/-</sup> mice. Thymocytes (a and b) or lymph node cells (c-f) from wild-type (a, c, and e) or CNA\alpha<sup>-/-</sup> (b, d, and f) mice of B6/129 background were stained with PE-CD4 and FITC-CD8 (a-d) or with PE-B220 and FITC-IgM (e and f).](image)
immunized subcutaneously with TNP-OVA (29). 10 d later, lymph node cells were harvested. The total number of lymph node cells and the percentages of CD4+ or CD8+ T cells were similar in the immunized wild-type and CNAα−/− mice (data not shown). After restimulation in vitro with OVA, T cells from wild-type mice proliferated much more rapidly than T cells from CNAα−/− mice (Fig. 3 a; data not shown). Addition of normal APCs or IL-2 to the in vitro cultures did not complement the proliferative defect of CNAα−/− T cells (Fig. 3 a).

The defect in the antigen-specific T cell response could be caused either by a defect in CNAα−/− T cells or by a defect in other cells whose function is required for the priming of antigen-specific T cells as a result of immunization. To distinguish between the two possibilities, we immunized CNAα−/−/RAG-2−/− chimeras with TNP-OVA and restimulated the lymph node cells with the immunogen after 10 d. CNAα−/− T cells from the CNAα−/−/RAG-2−/− chimeras did not proliferate as well in response to OVA (Fig. 3 b). In the CNAα−/−/RAG-2−/− chimeras, only T and B cells were completely CNAα−/−, while 90% of other cell types were derived from the RAG-2−/− blastocysts that were CNAα+/+, because the CNAα−/− ES-derived cells comprised only ~10% of the chimeric animals, as judged by coat color contributions. This suggested that the defective in vivo antigen-specific T cell response in CNAα−/− mice was caused by deficits in T cells per se, rather than deficits in any other cell types involved in the initiation of immune response.

Upon restimulation with OVA, the lymph node T cells from immunized CNAα−/− mice secreted significantly less IL-2 (average units of activity in wild type = 12.5 vs 1.5 U in CNAα−/−; P < 0.003) and IFN-γ (average units of activity in wild type = 513 vs 27 in CNAα−/−; P < 0.005) than the wild-type T cells. IL-2 and IFN-γ production by T cells from immunized wild-type and CNAα−/− mice. CNAα−/− T cells secreted significantly less IL-2 (average units of activity in wild type = 12.5 vs 1.5 U in CNAα−/−; P < 0.003) and IFN-γ (average units of activity in wild type = 513 vs 27 in CNAα−/−; P < 0.005) than the wild-type T cells. nd, not determined.

Upon restimulation with OVA, the lymph node T cells from immunized CNAα−/− mice secreted significantly less IL-2, IL-4, and IFN-γ than the lymph node T cells from immunized normal mice (Table 1). Primary antibody responses were also assessed in wild-type and CNAα−/− mice immunized with TNP-OVA. Similar titers of anti-TNP antibodies (IgG1 and IgG2a) were found in the serum of immunized wild-type and CNAα−/− mice (data not shown).

Table 1. IL-2 and IFN-γ secretion by Wild-type and CNAα−/− T Cells

| Mouse No. | Genotype | IL-2  | IFN-γ |
|-----------|----------|-------|-------|
| 2741      | +/+      | nd    | 799   |
| 2752      | +/+      | nd    | 982   |
| 2768      | +/+      | 7.6   | 622   |
| 2769      | +/+      | 7.5   | 33    |
| 2802      | +/+      | >16   | 537   |
| 2838      | +/+      | 15.4  | 358   |
| 2841      | +/+      | >16   | 257   |
| 2740      | −/−      | nd    | 0     |
| 2751      | −/−      | nd    | 0     |
| 2736      | −/−      | 0.7   | 164   |
| 2764      | −/−      | 0.7   | 0     |
| 2806      | −/−      | 4.2   | 0     |
| 2791      | −/−      | 0.4   | 0     |

CNAα−/− T Cells Respond Normally to Mitogens In Vitro and Remain Sensitive to CsA and FK506. We tested the responses of wild-type and CNAα−/− T cells to mitogenic stimulation with PMA plus ionomycin, ConA, and anti-CD3ε antibodies. All three mitogens induced the CNAα−/− IL-2 and IFN-γ production by T cells from immunized wild-type and CNAα−/− mice. CNAα−/− T cells secreted significantly less IL-2 (average units of activity in wild type = 12.5 vs 1.5 U in CNAα−/−; P < 0.003) and IFN-γ (average units of activity in wild type = 513 vs 27 in CNAα−/−; P < 0.005) than the wild-type T cells. nd, not determined.

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IL-2 and IFN-γ production by T cells from immunized wild-type and CNAα−/− mice. CNAα−/− T cells secreted significantly less IL-2 (average units of activity in wild type = 12.5 vs 1.5 U in CNAα−/−; P < 0.003) and IFN-γ (average units of activity in wild type = 513 vs 27 in CNAα−/−; P < 0.005) than the wild-type T cells. nd, not determined.

Calcineurin, especially calcineurin containing the α subunit, has been implicated as the target for the immunosuppressive drugs CsA and FK506, which inhibit TCR-mediated proliferation and IL-2 production by normal T cells (33–36). We tested the drug sensitivity of the CNAα−/− T cells. Cell proliferation and IL-2 and IL-4 production were inhibited in CNAα−/− and wild-type T cells stimulated

Figure 3. Antigen-specific proliferative response of T cells measured by [3H]thymidine incorporation. All experiments were done in triplicate. (a) After wild-type (open symbols) and CNAα−/− (closed symbols) mice were immunized (Materials and Methods), lymph node cells were restimulated with OVA alone (squares); OVA plus 4 × 10^4 T cell-depleted and irradiated C3HBL/6 spleen cells as exogenous APCs (diamonds); or with OVA plus 10 U/ml exogenous IL-2 (circle). (b) After wild-type (open squares) and CNAα−/−/RAG-2−/− chimeric (closed squares) mice were immunized, lymph node cells were restimulated with OVA.
Figure 4. Response to mitogenic stimulation. Spleen cells and lymph node cells from two wild-type (clear columns) and two CNAαnull (filled columns) mice of Black Swiss/129 background were stimulated with medium alone (−), 2.5 ng/ml PMA + 75 ng/ml ionomycin (P+I), 2.5 μg/ml Con A, soluble αCD3ε antibody (sCD3), or plate-bound αCD3ε antibody (x-CD3). 60 h after stimulation, cultures were pulsed with [3H]thymidine for 6 h. Experiments were done in duplicate.

with PMA plus ionomycin, ConA, or αCD3ε antibody, CNAαnull T cells, and wild-type T cells were both inhibited by CsA and FK506, as measured by proliferation and by IL-2 and IL-4 production (Fig. 5; data not shown). CNAαnull T cells were even more sensitive to these drugs than normal T cells, with a IC₅₀ two- to sevenfold lower than that of the wild-type T cells.

Discussion

We have generated mice that lack CNAα by inactivating the CNAα gene (Fig. 1). We demonstrate here that there was >65% reduction of calcineurin activity in the CNAαnull T lymphocytes. The residual calcineurin-like activity in the mutant T cells is likely to be contributed by other calcineurin isofoms or by other related phosphatases. Our data suggest, however, that α is the predominant isofom in T cells; in the absence of CNAα, expression of CNAβ is not increased (data not shown).

T and B cell development appears to proceed normally in CNAαnull mice (Fig. 2; data not shown), indicating that a functional CNAα gene is not required for the maturation of these cells. The mutant mice have a defective T cell response to antigen in vivo. Because this defective T cell response could not be complemented by other cells supplied by RAG-2null mice, we conclude that CNAα is required for in vivo antigen-specific T cell responses.

Even though CNAα is required for in vivo T cell response, it is not required for in vitro T cell responses to mitogens (Fig. 3 and 4; data not shown). This result contrasts with previous findings implicating CNAα in TCR-mediated proliferation and IL-2 production (33–36). We do not know the reason for the discrepancy, but our findings do suggest that in vitro T cell responses to αCD3ε antibody or ConA may not reflect the ability of T cells to respond to antigens in vivo.

CNAαnull T cells were sensitive to FK506 and CsA (Fig. 5; data not shown) suggesting that other molecules can mediate the immunosuppressive effect of these drugs. One of these proteins is most likely to be CNAβ. When CNAβ is over expressed in Jurkat cells, it has the same biological activities as over-expressed CNAα (O’Keeffe, S.J., unpublished results). Furthermore, 90–95% of the residual calcineurin activity found in CNAαnull T cells, which is most likely contributed by CNAβ, is FK506 sensitive. Both of these observations suggest that CNAβ can serve as an immunosuppressive drug target in the CNAαnull T cells. We cannot, however, rule out the possibility that there are other drug targets, since CsA and FK506 were shown to inhibit an antigen-specific response in the absence of a calcium signal (37). CNAαnull T cells had increased sensitivity to both CsA and FK506, suggesting that CNAα is the primary target of drug-immunophilin complexes, presumably because of the predominant presence of the CNAα protein in T cells.

The finding that CNAαnull mice have a marked T cell deficiency suggests that CNAα may be a relevant calcineurin isofom that mediates immunosuppression after treatment with CsA or FK506. We suggest that CsA and FK506 also target other proteins, which are sufficient to mediate the in vitro T cell responses to mitogens. However, these other CsA and FK506 target proteins cannot replace CNAα’s function in vivo. Furthermore, studies of CNAαnull mice should elucidate other roles for CNAα in physiology and pathobiology.

Figure 5. Proliferation of CNAαnull and wild-type T cells in the presence of CsA and FK506. 2 × 10⁴ wild-type (clear diamonds) or CNAαnull (solid diamonds) lymph node cells were stimulated with 10 ng/ml PMA + 300 ng/ml ionomycin in the presence of different concentrations of CsA (a) or FK506 (b). 60 h after stimulation, cultures were pulsed with [3H]thymidine for 6 h. Experiments were done in triplicate.
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