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Apoptosis-Linked Gene 2-Deficient Mice Exhibit Normal T-Cell Development and Function

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The apoptosis-linked gene product, ALG-2, is a member of the family of intracellular Ca^{2+}-binding proteins and a part of the apoptotic machinery controlled by T-cell receptor (TCR), Fas, and glucocorticoid signals. To explore the physiologic function of ALG-2 in T-cell development and function, we generated mice harboring a null mutation in the *alg-2* gene. The *alg-2* null mutant mice were viable and fertile and showed neither gross developmental abnormality nor immune dysfunction. Analyses of apoptotic responses of ALG-2-deficient T cells demonstrated that ALG-2 deficiency failed to block apoptosis induced by TCR, Fas, or dexamethasone signals. These findings indicate that ALG-2 is physiologically dispensable for apoptotic responses induced by the above signaling pathways and suggest that other functionally redundant proteins might exist in mammalian cells.

Apoptosis is a unique biological process of multicellular organisms and one of the driving forces underlying tissue/organ remodeling during animal development. This process also plays an important role during immune system development and function (1). Under physiological conditions, apoptotic response of lymphocytes is elicited by specialized molecular networks comprising of multiple signaling pathways and appears to be pivotal for shaping lymphocyte repertoires and preventing autoimmune diseases (8). Although large numbers of molecules involved in such networks have been identified recently (28), regulation and coordination between these molecules during immune system development and function remain elusive.

ALG-2 is a 22-kDa Ca^{2+}-binding protein belonging to the penta-EF (PEF) hand protein family that contains the Ca^{2+}-binding helix-loop-helix structure (14, 15, 25). The PEF hand protein family includes peflin, sorcin, and grancalcin, as well as the large and small subunits of calpains (2, 10, 15, 24). ALG-2 is ubiquitously expressed in mouse tissues, with its highest level of expression detected in thymus and liver. Recent data from T-cell lines indicate that ALG-2 protein plays a critical role for T-cell receptor (TCR)-, Fas-, and glucocorticoid-induced apoptosis, because depletion of ALG-2 in these cells blocks apoptosis whereas its overexpression significantly promotes apoptosis induced by these signals (5, 25). Although it is unclear how ALG-2 affects apoptosis of lymphocytes, evidence indicates that ALG-2 becomes rapidly associated with ALG-2-interacting protein-1 (AIP-1), a proapoptotic protein in a Ca^{2+}-dependent manner, suggesting that the regulation is likely controlled by a secondary messenger, Ca^{2+} (17, 26). Detailed biochemical analysis also places ALG-2 downstream of the ICE/Ced-3 signaling cascade activated by TCR, Fas, and dexamethasone stimulation (11).

To study the function of ALG-2 under physiological conditions, we generated ALG-2-deficient mice by using the gene targeting approach. Our data indicate that the general development and survival of mutant mice, as well as their immune system development and differentiation, appear to be normal. Of interest, TCR-, Fas-, and dexamethasone-induced apoptosis of T cells does not seem to be significantly impaired in the absence of ALG-2, indicating that other functionally redundant proteins might exist in mammals.

MATERIALS AND METHODS

Generation of *alg-2*$^{-/-}$ mice. A 9.0-kbp *Eco*RI fragment containing exons 1 through 3 of the *alg-2* gene was first cloned in pGEM7 (Promega). The resulting vector was named pGEM7-*alg-2*(E). pGEM7-*alg-2*(1.2)-Neo containing the short arm of the *alg-2* homologous region was constructed by the insertion of a phosphotransferase (*Neo*^{r}) cassette from pL2-Neo(2) (*Xho*I-*Bam*HI fragment) into pGEM7-*alg-2*(E) after pGEM7-*alg-2*(E) was cut with *Sma*I and *Bam*HI. pGEM7-*alg-2*(4.5) containing a 4.5-kbp *Sma*I-*Xho*I fragment of the *alg-2* gene was ligated with herpes simplex virus thymidine kinase (TK) cassettes to generate the pGEM7-*alg-2*(4.5)-TK plasmid. The *alg-2*(4.5)-TK fragment, recovered as a modified *Cla*I-*Bam*HI fragment, was ligated with a modified *Sal*I-*Bam*HI fragment of pTZ-*alg-2*(1.2)-Neo to obtain the final targeting vector, pGEM7-*alg-2*(T) (Fig. 1A).

Linearized targeting vector was introduced into embryonic stem (ES) cells by electroporation. The transfected cells were selected in G418- and ganciclovir-containing medium according to standard procedures (20). Homologous recombinants were identified by PCR analysis. The chimeric mice were first bred to C57BL/6J mice, and the germ line-transmitted heterozygous mice were further intercrossed to obtain *alg-2*$^{-/-}$ mice. PCR analysis was performed to determine genotype of mice using tail DNA samples. PCR primers used were a sense strand oligonucleotide (5′-GGC AAA GAT GTA GGA GGC GG-3′) and an anti-sense oligonucleotide (5′-CGC TGG AAG ACG TTC CAC-3′). Mice were maintained under specific-pathogen-free conditions at the Twinbrook II animal facility (National Institutes of Health).

Western blot analysis. Thymocyte lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred to a polyvinylidene difluoride membrane (Invitrogen). Membrane was blocked in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBST) and 5% fat-free milk

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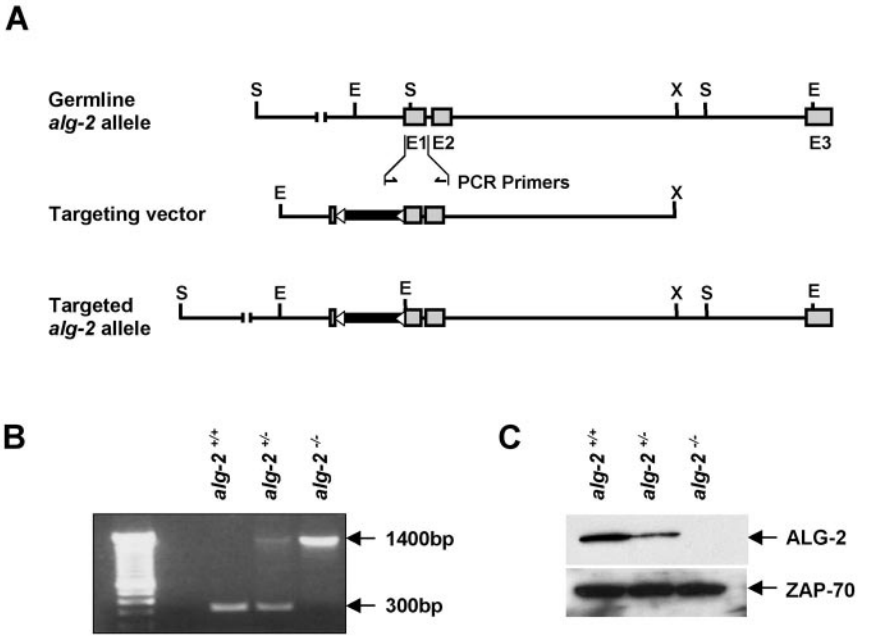

FIG. 1. (A) Strategy of *alg-2* gene targeting. The organizations of the wild-type *alg-2* gene, the targeting vector, and the targeted allele are depicted. The positions of exons 1 to 3 of the *alg-2* gene are shown (E1, E2, and E3). E, *Eco*RI; S, *Sma*I; X, *Xho*I. The locations of primers used for PCR are indicated. (B) PCR analysis of tail DNA from *alg-2* wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice. (C) Western blot analysis of ALG-2 protein in thymocytes. The lysate prepared from thymocytes of each mouse was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. ALG-2 protein was visualized using rabbit anti-ALG-2 polyclonal antibody. The same blot was reprobed with anti-Zap70 monoclonal antibody for quantification of protein loading.

for 1 h and then incubated overnight with rabbit anti-ALG-2 polyclonal antibody at 4°C. Horseradish peroxidase-conjugated anti-rabbit immunoglobulin and the enhanced chemiluminescence system (ECL system; Pierce) were used for protein visualization. To quantify the protein loading, the same blot was reprobed with anti-ZAP70 antibody (clone 29; Transduction Laboratory).

Flow cytometry and antibodies. Single-cell suspensions were prepared from thymus, spleen, and lymph nodes from 6- to 8-week-old *alg-2*$^{-/-}$ and wild-type littermates. Cells (10^6) were stained with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies specific for cell surface markers, CD4 (clone GK1.5), CD8 (clone 53-6.7), CD3 (clone 145-2C11), B220 (clone RA3-6B2) (BD Sciences), and HY TCR (clone C3.70), and the stained cells were analyzed by FACScan (Becton Dickinson) using Flowjo software (Tree Star, Inc.).

Cell proliferation assay and IL-2 measurement. For the T-cell proliferation assay, 10^5 splenic and lymph node T cells were cultured in triplicate in a final volume of 100 μl of RPMI medium supplemented with glutamine, 2-mercaptoethanol, and 10% fetal bovine serum for 2 days in the presence of various concentrations of anti-CD3 antibody, followed by incubation with [^{3}H]thymidine (0.5 μCi/well; Amersham Pharmacia) for 16 h. Cells were then harvested using a cell harvester, and incorporated radioactivity was measured with a scintillation counter. The amount of interleukin-2 (IL-2) in the cell culture supernatant was determined using an IL-2 immunoassay kit according to the protocol provided by the manufacturer (R&D Systems).

In vitro and in vivo analyses of apoptosis. Cells were stimulated according to the following conditions. For thymocyte apoptosis, 5×10^6 thymocytes were stimulated with plate-bound anti-CD3 antibody (clone 145-2.C11; 10 μg/ml) in the presence of anti-CD28 antibody (clone 37.51; 10 μg/ml), anti-Fas antibody (clone Jo2, 1 μg/ml; BD Science), or dexamethasone (10^{-7} M) for 24 h. The percentage of apoptotic cells was determined either by staining with propidium iodide (PI) and Annexin V and analyzing on FACScan or by gel electrophoresis (7) to quantify DNA fragmentation. For the in vivo apoptosis assay, mice were administered with 10 μg of a purified hamster monoclonal anti-Fas antibody intravenously. Mouse lethality was monitored over 8 h.

RT-PCR. Cellular RNA was isolated from thymocytes and purified CD4 T cells using RNAzol B (TEL-TEST, Inc). First, strand cDNA was synthesized using an oligo(dT) primer, and 2 μl of cDNA of each sample was used in PCR. The reaction was carried out in 50 μl of reaction buffer containing 1 U of *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 20 mM Tris (pH 8.4), and 50 mM KCl and was performed for a total of 31 cycles under cycling conditions: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Gene-specific primers used were sense strand oligonucleotide (5′-GCC CTA GAG TAG CAA TGG AGT TAG-3′) and anti-sense oligonucleotide (5′-TAC AAT GTT GGC AGT AGG ATG TG-3′) for peflin, sense strand oligonucleotide (5′-GGT TGA TAA AGA CAG GAG TGG AGT-3′) and anti-sense oligonucleotide (5′-GAT GTC ATG GAA CTG ATC AGA GAG-3′) for ALG-2, and sense strand oligonucleotide (5′-GGA CTC CTA TGT GGG TGA CGA GG-3′) and anti-sense oligonucleotide (5′-GGG AGA GCA TAG CCC TCG TAG AT-3′) for β-actin. PCR amplification products were analyzed by gel electrophoresis using 1.5% Tris-acetate-EDTA (TAE) agarose gel.

RESULTS

Generation of *alg-2*$^{-/-}$ mice. The gene targeting vector depicted in Fig. 1A was used to disrupt the *alg-2* gene in ES cells, and homologous recombinants were identified by PCR. Five independent ES cell clones carrying the *alg-2* gene mutation were obtained, one of which was used to generate chimeric mice. Homozygous (*alg-2*$^{-/-}$) mutant mice were identified by PCR (Fig. 1B). Inactivation of the *alg-2* gene was confirmed by Western blot and Northern blot analyses (Fig. 1C and data not shown). The *alg-2*$^{-/-}$ mice were fertile and healthy, and inspection of various organs in the mutant mice revealed no abnormality. Offspring obtained from the *alg-2*$^{+/-}$ × *alg-2*$^{+/-}$ mating showed the expected 1:2:1 ratio of *alg-2*$^{+/+}$, *alg-2*$^{+/-}$, and *alg-2*$^{-/-}$ genotypes. These results indicate that *alg-2* is not required for the general development and survival of mice.

Lymphocyte development is normal in ALG-2-deficient

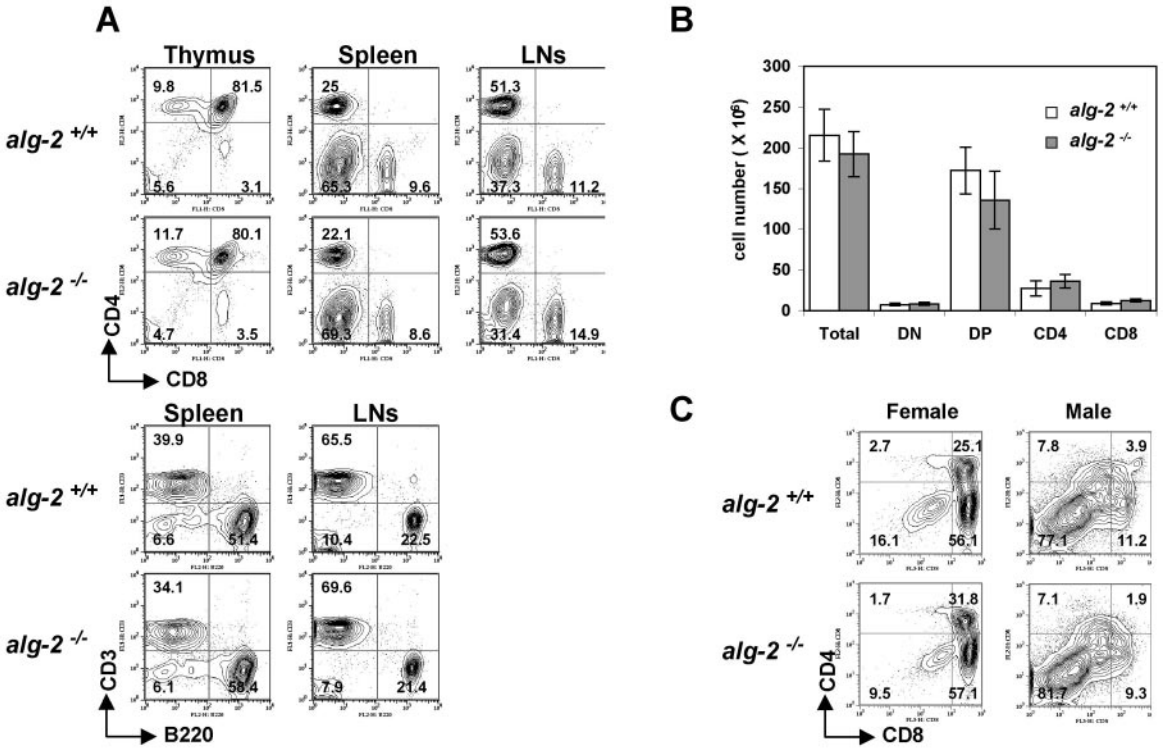

FIG. 2. Normal lymphocyte populations in the thymus, spleen, and lymph nodes. (A) The thymus, spleen, and lymph nodes (LNs) were isolated from 6- to 8-week-old control or *alg-2*$^{-/-}$ mice. Cells were stained with PE-conjugated anti-CD4 antibody and FITC-conjugated anti-CD8 antibody or FITC-conjugated anti-CD3 antibody and PE-conjugated anti-B220 antibody. The contour plots represent results of more than three independent experiments. (B) Absolute numbers of thymocyte subsets. Bars represent mean values of each cell population in thymus. For wild-type mice, $n = 4$; for *alg-2*$^{-/-}$ mice, $n = 3$. (C) Positive and negative selection of *alg-2*$^{-/-}$ thymocytes. Thymocytes were from 6- to 8-week-old HY TCR transgenic wild-type and *alg-2*$^{-/-}$ mice. Shown are gated HY transgene-expressing (C3.70-positive) cells. The numbers shown in each quadrant are percentages of corresponding cell populations.

mice. To analyze the possible influence of ALG-2 deficiency on lymphocyte development, we compared the cellularities of thymus, spleen, and lymph nodes of *alg-2*$^{-/-}$ and wild-type mice by flow cytometry analysis. Total numbers of nucleated cells in thymus, spleen, and lymph nodes were the same between the *alg-2* mutant and wild-type mice. Analysis of thymocytes revealed similar frequencies of $CD4^-CD8^-$ (DN), $CD4^+CD8^+$(DP), $CD4^+$, and $CD8^+$ cells between mutant and wild-type mice (Fig. 2A and B). In both spleen and lymph node of the *alg-2*$^{-/-}$ mice, the $CD4^+$, $CD8^+$, or $B220^+$ cell populations also appeared to be unaltered compared with those of wild-type mice (Fig. 2A). To determine whether *alg-2* mutation influences development of the T-cell repertoire, we bred *alg-2*$^{-/-}$ mice with HY TCR transgenic mice, which express TCR recognizing the male specific antigen HY in the context of major histocompatibility complex class I (MHC-I). In this model system, in the presence of MHC-I H-2D^b molecules, transgenic TCR-positive $CD8^+$ T cells are positively selected (positive selection) in female mice, whereas in male mice, this T-cell population is depleted (negative selection) via TCR-mediated apoptosis (27). As shown in Fig. 2C, $CD8^+$ T cells were depleted in thymus of male *alg-2*$^{-/-}$ mice as efficiently as in thymus of wild-type animals, demonstrating that negative selection was not impaired in the mutant mice. In the case of the female mice, almost equal numbers of $CD8^+$ T cells were generated in the *alg-2*$^{-/-}$ thymus and wild-type thymus, indicating that positive selection of $CD8^+$ T cells remained normal in the mutant mice. Taken together, these results demonstrated that the development of both T- and B-lineage cells was not affected in the absence of ALG-2 protein.

T-cell function remains normal. To examine the impact of ALG-2 deficiency on mature T-cell function, we examined T-cell proliferation and IL-2 production after anti-CD3 antibody cross-linking in vitro. Splenic and lymph node T cells isolated from 8-week-old wild-type and *alg-2*$^{-/-}$ mice exhibited similar antibody dose-dependent proliferation (Fig. 3A). Similarly, the levels of IL-2 secretion by the ALG-2-deficient and wild-type T cells were also indistinguishable (Fig. 3B). These results suggest that ALG-2 is not required for mature T-cell activation in vitro.

T cells from ALG-2-deficient mice retain susceptibility to apoptotic stimuli. Receptor-triggered apoptosis is known to be essential for the generation of a functional T-cell repertoire and proper control of immune responses (18). It is believed that autoreactive immature thymocytes are eliminated by a TCR- and/or Fas-induced apoptotic mechanism, whereas cells expressing TCR with low affinity to MHC are aborted, possibly in a glucocorticoid-dependent manner (23). Glucocorticoid receptor signaling is also responsible for apoptosis of thymocytes when mice are under stress (6). It is suggested that ALG-2 may function as a proapoptotic signaling protein downstream of the

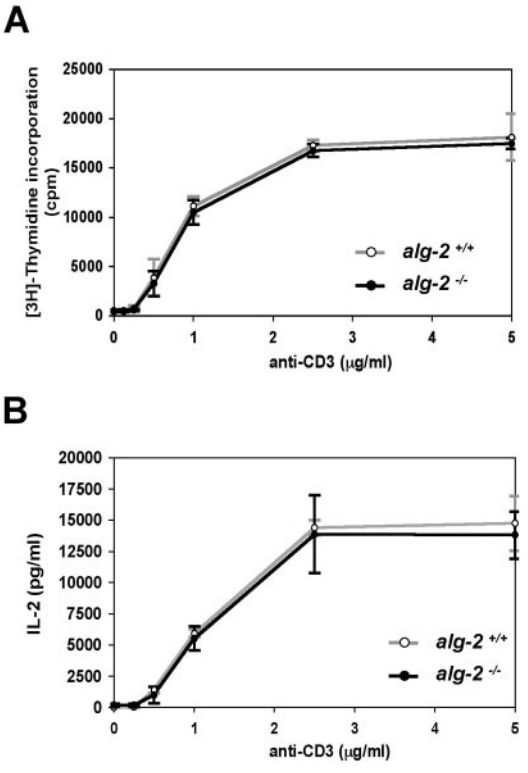

FIG. 3. T-cell proliferation and IL-2 production. (A) Anti-CD3 antibody-induced T-cell proliferation. Splenic and lymph node T cells were isolated from 8-week-old mice and stimulated with plate-bound anti-CD3 antibody for 2 days. Shown are results from triplicate samples in each experiment. Data are representative of three independent assays. (B) IL-2 secretion after anti-CD3 antibody stimulation. Data represent triplicate samples and are representative of two independent assays.

signaling pathway induced by TCR, Fas, and glucocorticoid in T cells (5, 25). To determine whether ALG-2 is involved in receptor-mediated signaling during thymocyte development, we examined apoptotic responses of *alg-2*$^{-/-}$ thymocytes after anti-TCR and Fas receptor triggering or treatment with dexamethasone. Apoptotic cells were stained by PI/Annexin V and analyzed by flow cytometry or measured by DNA fragmentation using a gel assay. We found that all three stimuli induced the same degree of apoptosis, as a similar staining pattern of PI/Annexin V-positive cells as well as a similar extent of DNA fragmentation characteristic for apoptotic cells were observed in the treated *alg-2*$^{-/-}$ and wild-type thymocytes (Fig. 4A). These results indicate that ALG-2 is not essential for apoptosis induction of immature thymocytes during thymic selection.

During T-cell activation, repeated stimulation of activated T cells through TCR in the presence of IL-2 may elicit an apoptotic response known as activation-induced cell death (21), and this process has been proposed to control proper immune responses and to prevent autoimmunity. Because this process is regulated by TCR signal, we decided to examine whether ALG-2 deficiency blocks mature T-cell apoptosis after repeated triggering through TCR. Mature T cells from wild-type or *alg-2*$^{-/-}$ mice were first stimulated with ConA and subsequently cultured for several days in the presence of IL-2. Cells were then rechallenged with anti-CD3 antibody, and apoptotic responses were measured by DNA fragmentation assay. As shown in Fig. 4B, *alg-2*$^{-/-}$ mutant and wild-type T cells exhibited indistinguishable patterns of DNA fragmentation after stimulation. Thus, we conclude that deletion of the *alg-2* gene does not impose any apparent effect on activation-induced T-cell death in vitro.

ALG-2-deficient mice are susceptible to anti-Fas antibody administration. Because ALG-2-deficient T cells did not show any sign of resistance to apoptosis induced by TCR, Fas, and dexamethasone, we decided to examine whether this protein is involved in Fas-induced cytolytic activity of other somatic cells. It has been shown that after in vivo administration of anti-Fas antibody (Jo2), mice become moribund within several hours mainly due to liver damage (19). In contrast, mice with mutations that inhibit the Fas-mediated signaling pathway exhibit resistance to such treatment (12, 29). To test whether ALG-2-deficient mice become resistant to anti-Fas treatment, 10 μg of Jo2 antibody was intravenously injected into *alg-2*$^{-/-}$ mutant and wild-type mice and death was monitored at 30-min intervals after injection. The death curves of *alg-2*$^{-/-}$ and wild-type mice were nearly identical (Fig. 5), indicating that ALG-2 is not required for Fas-induced apoptosis of liver cells in vivo.

DISCUSSION

It has been demonstrated previously that suppression of ALG-2 expression in T-cell lines blocked TCR-induced apoptosis of T cells. Further evidence indicates that ALG-2 may function to induce apoptosis in a Ca^{2+}-dependent manner (25, 26). Recently, Jung and his group observed that ALG-2 directly interacts with the death domain of Fas and that anti-Fas antibody triggering promotes cleavage of ALG-2 and release in the cytosol of a cleaved ALG-2 product, suggesting a role of ALG-2 in Fas signaling (9). In an attempt to study the physiological importance of ALG-2, we generated *alg-2*$^{-/-}$ mice by gene targeting. We found that *alg-2*$^{-/-}$ mice did not exhibit any abnormality in terms of development, reproduction, and life span. Immune system development and function also appeared normal in the absence of ALG-2. In particular, the mutant mice had normal thymocyte development, and function of mature mutant T cells did not seem to be altered compared to that of wild-type T cells. Consistent with these observations, our apoptosis analyses indicated that both immature thymocytes and mature T cells from *alg-2*$^{-/-}$ mice retained their susceptibility to apoptotic stimuli, such as anti-TCR and -Fas antibody stimulation or dexamethasone treatment. These results together indicate that ALG-2 is dispensable for animal development and survival, as well as for immune system development and function.

ALG-2 was initially identified as a proapoptotic protein from a T-cell line, because expression of its anti-sense-chain cDNA blocked TCR-induced apoptosis (25) whereas, in contrast, overexpression of this protein promoted apoptosis. There are three possible explanations for the lack of a similar effect in *alg-2*$^{-/-}$ T cells. First, although ALG-2 protein is absent in mutant cells, it is likely that other functionally redundant proteins may take over the role of ALG-2 in the signaling cascade. Our analysis revealed that peflin, a PEF hand-containing protein with the highest homology to ALG-2 (10), was indeed

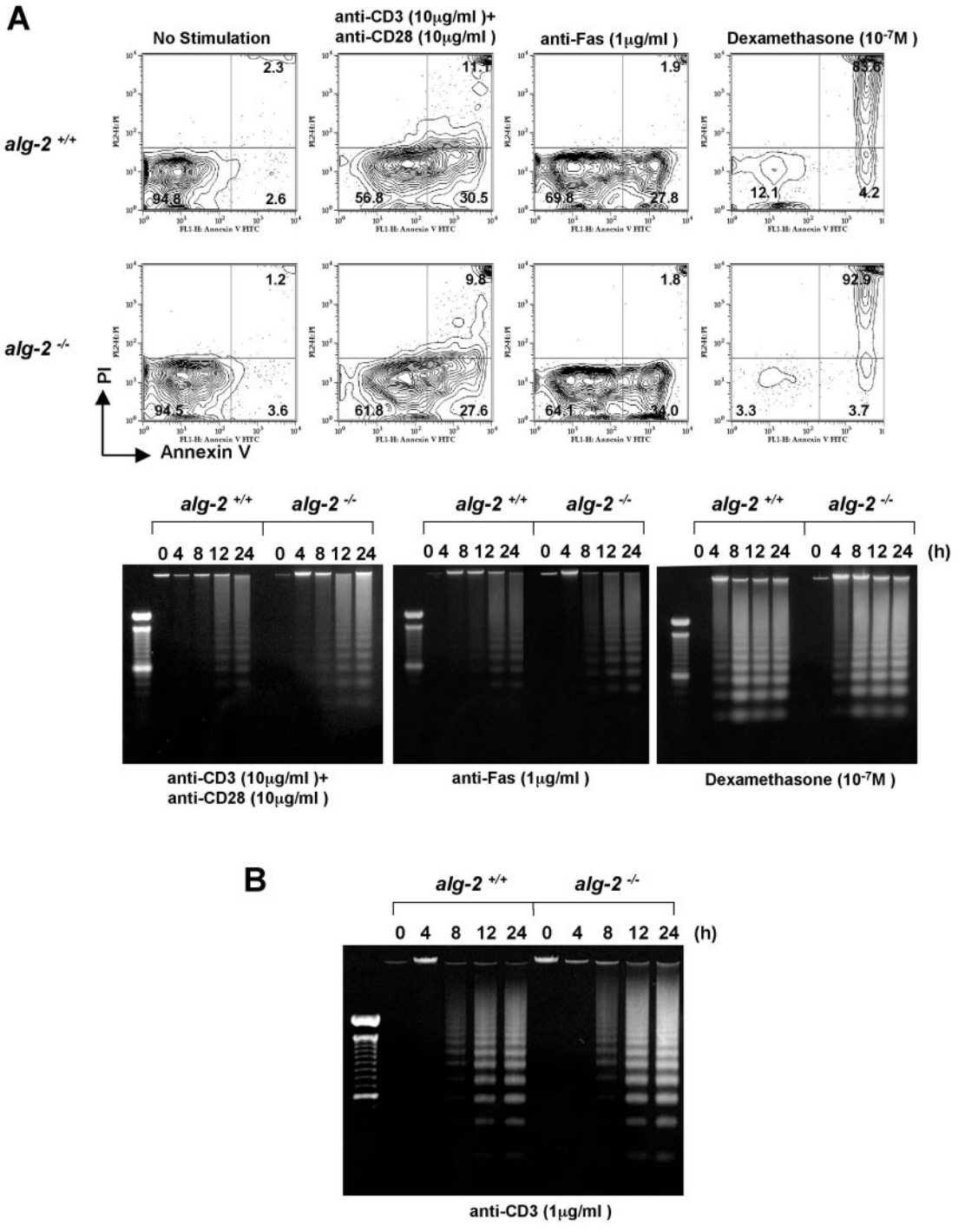

FIG. 4. Apoptosis of thymocytes and peripheral T cells in response to anti-CD3 antibody, anti-Fas antibody, and dexamethasone treatment. (A) PI/Annexin V staining of apoptotic cells (above) and analysis of DNA fragmentation (below) in thymocytes after 24 h of treatments. Thymocytes were cultured in the presence or absence of the indicated stimuli. (B) DNA fragmentation assay of activation-induced T-cell apoptosis. A molecular weight marker (100-bp DNA ladders) is shown in the left lane.

expressed in mutant thymocytes and peripheral $CD4^+$ T cells (Fig. 6). Second, because the previously observed proapoptotic effect of ALG-2 was obtained from an in vitro-cultured cell line, we cannot exclude the possibility that this cell line might carry alterations in other genes that coordinate with ALG-2 during apoptosis induction. The third possibility is that the initial evidence of ALG-2 involvement in T-cell apoptosis using the anti-sense cDNA approach is not completely accurate, because anti-sense cDNA may block the expression of more than one gene product; however, this possibility seems less likely, because overexpression of ALG-2 protein may promote TCR-induced apoptosis.

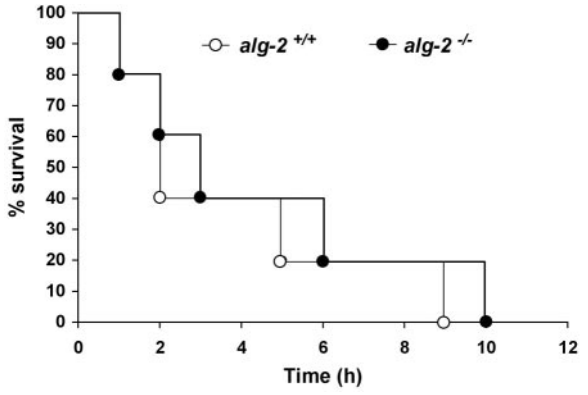

FIG. 5. Anti-Fas antibody-induced death of mice. Animal mortality was monitored every 30 min for 8 h. Each treatment included five mice per group. Mice were injected intravenously with 10 μg of anti-Fas (Jo2) antibody diluted in 200 μl of PBS.

Although ALG-2 deficiency did not yield the expected phenotype for development and function of immune system, its involvement in apoptotic processes of other somatic cells cannot be excluded. The glioma-associated protein SETA, which also interacts with AIP to form a multimolecular complex, has been demonstrated to modulate cell death in astrocytes (3). Moreover, expression of ALG-2 is significantly upregulated, together with a broad profile of other gene products, in brain in response to ischemia (13). Together, these findings suggest that ALG-2 needs to be incorporated into a genetic program and that its coordination with other factors might be necessary to execute the proapoptotic function.

Although biological functions of PEF domain containing proteins, including ALG-2, calpain, peflin, sorcin, and grancalcin, are poorly understood, their different expression and association partners suggest that they might have diverse functions. Recent evidence suggests that sorcin might be involved in the regulation of cardiac muscle contraction (16), whereas calpain functions as a protease and likely regulates cell adhesion (4, 22). Thus, mice deficient in ALG-2 should prove to be a useful model in which to explore the exact biological function of ALG-2 from other perspectives.

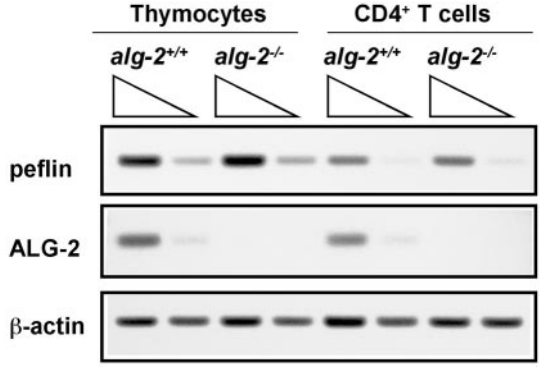

FIG. 6. Expression of peflin in *alg-2*$^{-/-}$ thymocytes and peripheral CD4$^+$ T cells. Cellular RNA from thymocytes and peripheral CD4$^+$ T cells was extracted and reverse transcribed. Samples were serially diluted (1:4), and PCR was performed using primers specific for peflin, ALG-2, and β-actin. Data are representative of at least three independent experiments.

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