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Role of DNA 5-Methylcytosine Transferase in Cell Transformation by *fos*

A. V. Bakin and T. Curran*

The Fos and Jun oncoproteins form dimeric complexes that stimulate transcription of genes containing activator protein–1 regulatory elements. We found, by representational difference analysis, that expression of DNA 5-methylcytosine transferase (*dnmt1*) in *fos*-transformed cells is three times the expression in normal fibroblasts and that *fos*-transformed cells contain about 20 percent more 5-methylcytosine than normal fibroblasts. Transfection of the gene encoding Dnmt1 induced morphological transformation, whereas inhibition of *dnmt1* expression or activity resulted in reversion of *fos* transformation. Inhibition of histone deacetylase, which associates with methylated DNA, also caused reversion. These results suggest that *fos* may transform cells through alterations in DNA methylation and in histone deacetylation.

The *fos* proto-oncogene (c-*fos*) is the cellular homolog of the oncogene that is carried by the Finkel-Biskis-Jinkins (FBJ) murine sarcoma virus (*1*). Its protein product, Fos, is a nuclear phosphoprotein that forms heterodimeric complexes with Jun and activating transcription factor/cyclic adenosine 3′,5′-monophosphate (cAMP) response element–binding protein family members and regulates transcription through activator protein–1 (AP1) and cAMP-responsive elements (*1*). Fos can be induced rapidly and transiently in many cells by diverse extracellular stimuli (*2*). It is believed to function in a transcription factor network that couples extracellular stimuli to alterations in gene expression. Gene disruption studies have shown that Fos is required for differentiation of osteoclasts, for oncogenic conversion of phorbol ester–induced skin tumors, and for light-induced death of photoreceptor cells in the eye (*3*). It is likely that the many heterodimeric complexes containing Fos regulate distinct target genes in different physiological contexts. Continuous expression of *fos* results in the morphological transformation of cultured rodent fibroblasts in vitro and the induction of bone tumors in mice (*1, 4*). Cell transformation requires the leucine-zipper and the DNA binding regions of *fos* (*5*). In transformed rat fibroblasts, the major partner of Fos is c-Jun, which was first described as the Fos-binding protein, p39 (*6*). Using a conditional expression system based on LacI, we demonstrated that a continuous period (~72 hours) of c-*fos* expression is required for complete morphological conversion, which occurs even in quiescent cells (*7*). Like other oncogenic transcription factors, *fos* is thought to transform cells by inappropriate regulation of gene expression (*7, 8*).

To identify target genes of *fos* that are effectors of cell transformation, we used representational difference analysis (RDA) (*9*) to isolate mRNA species, which are present in *fos*-transformed cells at levels that are higher than those present in normal fibroblasts. cDNA was prepared from LacIc-*fos* cells, which contain a *fos* gene regulated by the LacI activator protein [comprising LacI, a nuclear localization signal, and the VP16 activator (*7*)], in the presence (normal phenotype) and absence (transformed phenotype) of isopropyl-β-D-thiogalactopyranoside (IPTG). In these cells, *fos* is rapidly repressed in the presence of IPTG and is rapidly induced in its absence. Although several *fos* target genes are induced within minutes of *fos* expression in these cells, transformation requires 2 to 3 days. The cDNA populations obtained from transformed and normal cells were used in RDA as tester and driver, respectively (*10*).

The difference products that were obtained after three rounds were analyzed by DNA sequencing and included the cathepsin L and aquaporin-1 genes (Fig. 1A) that were previously shown to be regulated by Fos or to contain AP1 regulatory elements (*11, 12*). In transformed cells, the candidate target genes were

Department of Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN 38105, USA.

*To whom correspondence should be addressed. E-mail: fos1@aol.com

Table 1. Effect of *fos* transformation on the 5-methylcytosine content of DNA. The level of methylation relative to that in normal 208F cells (100%) was determined by high-performance liquid chromatography (HPLC) analyses of genomic DNA digested to nucleosides (*26*). DNA (10 μg per sample) was digested with deoxyribonuclease I, phosphodiesterase, and alkaline phosphatase, and the products were separated by HPLC. The data represent the average of three independent experiments (±SD), except for data from the 0.5% serum samples, which are from two experiments. Dash, not applicable.

Cellular source of DNA	Time of culture (hours)	Methylation (%)
CMVc-*fos*	–	119 ± 4.9
FBJ/R	–	129 ± 4.7
LacIc-*fos* + IPTG	24	110 ± 4.1
LacIc-*fos* + IPTG	48	100 ± 5.9
LacIc-*fos* + IPTG	72	97 ± 3.6
LacIc-*fos* – IPTG	24	126 ± 4.1
LacIc-*fos* – IPTG	48	132 ± 8.6
LacIc-*fos* – IPTG	72	120 ± 2.4
LacIc-*fos* – IPTG in 0.5% serum	72	116 ± 4.3
LacIc-*fos* + IPTG in 0.5% serum	72	100 ± 3.8
CMV*dnmt1*	–	167 ± 7.6

expressed at levels that were 2 to 8 times the levels in control cells. The RDA products also included several novel sequences and four genes: *dnmt1*; breast cancer candidate gene-1 (*bcsc-1*) (this gene is no longer thought to be a strong breast cancer candidate gene); ribosomal protein S3 (*rps3*); and the gene encoding pyruvate kinase, which has not been previously shown to be regulated by *fos*. We examined the expression levels of *dnmt1*, *bcsc-1*, and cathepsin L by Northern (RNA) blot analysis of several independently derived *fos*-transformed cell lines and determined the time course of expression during transformation and reversion of LacIc-*fos* cells (Fig. 1A). Normal rat fibroblasts express c-*fos* at a low basal level, but this level was increased ~25-fold after transformation by a c-*fos* gene under the control of the cytomegalovirus (CMV) immediate early gene CMV promoter (CMVc-*fos*) (Fig. 1A). The v-*fos* gene in the FBJ/R construct is a highly transforming mutant (*13*) that is expressed at relatively low levels in transformed cells. In LacIc-*fos* cells, *fos* was expressed in the presence of IPTG at a level similar to that in 208F cells, but this level rapidly increased ~18-fold in the absence of IPTG (Fig. 1A). The expression patterns of the selected RDA clones paralleled the expression pattern of *fos* (Fig. 1A). No consistent fluctuations were observed in expression of control genes (β-actin, mitochondrial 16*S* rRNA, and 28*S* and 18*S* rRNA). Several other genes (including those encoding aquaporin-1, pyruvate kinase, *rps3*, annexin IV, and *rad21*) were expressed preferentially in *fos*-transformed cells. In contrast, other previously identified targets of *fos* [including the genes encoding ezrin, tropomyosin-1, and vascular endothelial growth factor–D (*8*)] were not differentially expressed in our *fos*-transformed cells.

In principle, genes that contribute to *fos* transformation should be (i) inducible by *fos*, (ii) present in all *fos*-transformed cells, (iii) able to encode stable products, (iv) required for *fos* transformation, and (v) capable of inducing transformation in the absence of *fos*. Of the nine genes that we identified by RDA, *dnmt1* was the best candidate to match these criteria. A 190–base pair (bp) fragment of the *dnmt1* gene, corresponding to regions 2108 through 2297 of the mouse *dnmt1* gene (GenBank accession number AF036009), was present in a composite RDA clone that also included the β-actin and *bcsc-1* genes.

Dnmt1, which catalyzes the transfer of methyl groups from *S*-adenosyl methionine to the C-5 position of cytosines in DNA, has been previously implicated in cancer; it is overexpressed in tumor cells, and it can induce transformation when introduced into fibroblasts (*14*). High levels of DNA methylation are associated with gene silencing, and progressive methylation of several genes has been observed in cells that overexpress *dnmt1* (*15*).

The 5.5-kb *dnmt1* mRNA was present in CMVc-*fos*– and FBJ/R-transformed cells at levels that were 2 to 3 times as high as the

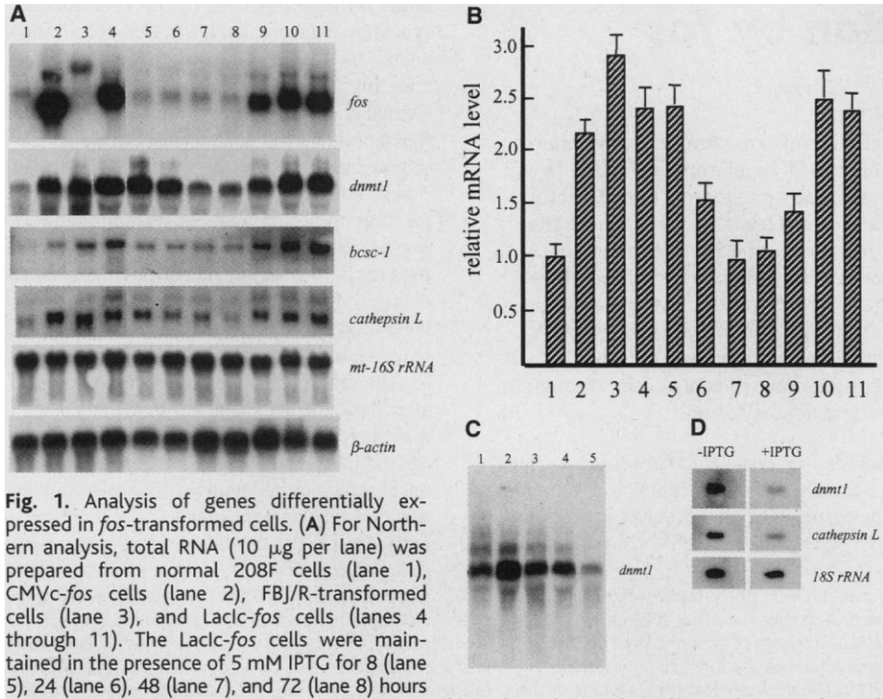

Fig. 1. Analysis of genes differentially expressed in *fos*-transformed cells. (**A**) For Northern analysis, total RNA (10 μg per lane) was prepared from normal 208F cells (lane 1), CMVc-*fos* cells (lane 2), FBJ/R-transformed cells (lane 3), and LacIc-*fos* cells (lanes 4 through 11). The LacIc-*fos* cells were maintained in the presence of 5 mM IPTG for 8 (lane 5), 24 (lane 6), 48 (lane 7), and 72 (lane 8) hours and were maintained in the absence of IPTG for 4 (lane 9), 24 (lane 10), 48 (lane 11) and 72 (lane 4) hours. Blots were hybridized with probes specific for c-*fos* (1164-bp fragment of rat cDNA), cathepsin L [1100-bp polymerase chain reaction (PCR) fragment of EST clone (GenBank accession number AA617608)], *bcsc*-1 (219-bp PCR fragment), and *dnmt1* (2138-bp fragment of mouse *dnmt1*). Probes for mitochondrial 16*S* rRNA (mt-16*S* rRNA) and β-actin were used as controls. (**B**) PhosphorImager quantitation of *dnmt1* mRNA levels in normal and *fos*-transformed cells. The level of *dnmt1* expression was normalized to the level of β-actin. The data represent the average of three independent experiments. Error bars indicate ±SD. (**C**) Northern blot analysis of *dnmt1* expression in *fos*-transformed cells that were maintained in 0.5% serum. Total RNA (10 μg per lane) was prepared from LacIc-*fos* cells that were cultured in the presence of 5mM IPTG for 72 hours in 0.5% serum (lane 1); from LacIc-*fos* cells that were cultured in the absence of IPTG for 24 (lane 2), 48 (lane 3), and 72 (lane 4) hours; and from normal 208F cells in 0.5% serum (lane 5). The filter was hybridized with a *dnmt1* probe. (**D**) Analysis of *dnmt1* and cathepsin L transcription in normal and *fos*-transformed cells by nuclear run-on. cDNA fragments (1 μg per slot, except for cDNA of 18*S* rRNA, which represented 0.2 μg) were hybridized with RNA probes prepared by transcription in nuclei from LacI-*fos* cells that were maintained for 72 hours in the absence (–IPTG) or presence of IPTG (+IPTG).

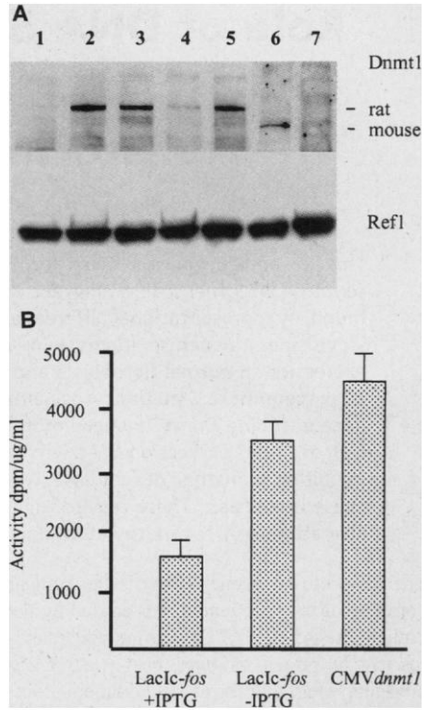

Fig. 2. Expression and activity of Dnmt1 in *fos*-transformed cells. (**A**) Immunoblot analysis of Dnmt1 levels. Proteins (40 μg per lane) from normal and *fos*-transformed cells were separated by electrophoresis on SDS-polyacrylamide gels, transferred onto filters, and treated with polyclonal antibodies to Dnmt1 or Ref1. Lanes are as follows: 208F cells (lane 1), CMVc-*fos* cells (lane 2), FBJ/R cells (lane 3), LacIc-*fos* cells plus IPTG (lane 4), LacIc-*fos* cells with no IPTG (lane 5), CMV*dnmt1* cells (lane 6), and CMVc-*fos* cells expressing antisense *dnmt1* (lane 7). (**B**) Analysis of Dnmt1 activity. Protein extracts (from LacIc-*fos* cells maintained for 72 hours in the presence or absence of IPTG and from 208F cells transfected with CMV*dnmt1*) were assayed for Dnmt1 activity by the incorporation of [^{3}H]-methyl into poly(dI-dC:dI-dC) as described (*14*). The data represent the average of three independent experiments. Error bars indicate ±SD.

levels in parental 208F fibroblasts (Fig. 1, A and B). Induction of c-*fos* expression by removal of IPTG increased *dnmt1* mRNA levels by ~1.5-fold within 4 hours and by about threefold by 48 hours (Fig. 1, A and B). Abrogation of c-*fos* expression by the addition of IPTG decreased the level of *dnmt1* mRNA. Although the increase in *dnmt1* expression was modest, a small increase in the steady state level of *dnmt1* can transform NIH3T3 cells (*14*), and there is only a threefold elevation in *dnmt1* levels in *ras*-transformed adrenocortical Y1 cells (*16*). The increase in *dnmt1* was not simply due to the proliferative state of *fos*-transformed cells, because the same increase occurred during the morphological conversion of serum-deprived fibroblasts by LacIc-*fos* (Fig. 1C).

The increase in *dnmt1* expression appears to be a consequence of an approximately threefold increase in its transcription rate as determined by nuclear run-on analyses (Fig. 1D). These data are consistent with the possibility that Fos regulates *dnmt1* transcription directly. In support of this hypothesis, a dominant-negative c-*jun* gene has been shown to reduce *dnmt1* expression in *ras*-transformed cells (*16*), and AP1 sites have been identified in a region of the *dnmt1* gene that activates transcription in a *ras*- and *jun*-dependent manner (*16, 17*).

Dnmt1 protein levels were elevated ~10-fold in three independent *fos*-transformed cell lines (CMVc-*fos*, FBJ/R-4, and LacIc-*fos* cells) (Fig. 2A). In LacIc-*fos* cells that were maintained in the presence of IPTG for 3 days, Dnmt1 protein levels were substantially reduced, although they remained about twofold higher than those in normal fibroblasts (Fig. 2A). In contrast, no changes were observed in the levels of Ref-1, a redox/DNA repair enzyme (*18*). Protein extracts from *fos*-transformed cells stimulated a twofold greater incorporation of [^{3}H]-methyl into poly(dI-dC:dI-dC) in comparison with normal cell extracts in vitro (Fig. 2B). Furthermore, DNA from *fos*-transformed cells contained ~20% more 5-methylcytosine than DNA from normal cells (Table 1). The 5-methylcytosine content of LacIc-*fos* cells increased 24 hours after the withdrawal of IPTG and reached a maximum after 48 hours. For comparison, rat cells transfected with a CMV*dnmt1* construct contained ~70% more 5-methylcytosine than normal cells (Table 1). These data are consistent with the range (20 to 100%) of the increased DNA methylation content that was reported for NIH3T3 cells and human fibroblasts transformed by *dnmt1* (*14*).

Fibroblasts transfected with CMV*dnmt1* exhibited a transformed cell morphology that was strikingly similar to that of *fos*-transformed cells (Fig. 3, E and F). To determine whether Dnmt1 is required for *fos*-mediated transformation, we inhibited its expression in *fos*-transformed cells with a *dnmt1* antisense vector, and we blocked its activity with 5-aza-deoxycytidine (5-aza-dC), an inhibitor of *dnmt1*. Stable cell lines expressing antisense *dnmt1* together with CMVc-*fos* exhibited a flat morphology and a contact inhibition phenotype, which were similar to that expressed in normal fibroblasts (Fig. 3G). Dnmt1 protein levels were substantially reduced in these cells, whereas Fos protein levels remained unchanged (Fig. 2A). Treatment of *fos*-transformed cells with 5-aza-dC for 5 days also resulted in a flatter cell morphology and contact inhibition; however, Fos expression was also reduced (Fig. 3H). Thus, elevated levels of Dnmt1 are required for the maintenance of the transformed cell phenotype that is induced by *fos*.

DNA methylation causes repression of gene expression by promoting the condensation of chromatin. Methylated sites on DNA bind the 5-methylcytosine binding protein (MeCP2), which exists in a complex with Sin3A and histone deacetylase (HDAC) (*19*). This complex decreases the levels of histone acetylation, resulting in a compact chromatin structure. To investigate whether *fos* transformation requires histone deacetylation activity, we used the HDAC inhibitor trichostatin A (TSA) (*20*). Treatment of both *fos*- and CMV*dnmt1*-transformed cells with TSA for 3 days induced a normal cell morphology (Fig. 3, J and K). These results suggest that *fos* transformation is mediated by elevated expression of *dnmt1*, which represses gene expression (possibly by the increased recruitment of HDAC to methylated DNA). TSA and other HDAC inhibitors also cause reversion of *ras*-transformed cells (*21*).

Paradoxically, many tumors that overexpress *dnmt1* have hypomethylated genomic DNA (*15*). Furthermore, whereas hypomethylation has been suggested as contributing to colon cancer in humans (*22*), suppression of *dnmt1* reduces the incidence of intestinal polyps in *Min* mice (23). These apparent contradictions can be resolved by considering the patterns of DNA methylation in tumor cells. Increased expression of *dnmt1* is associated with regional areas of hypermethylation in CpG islands

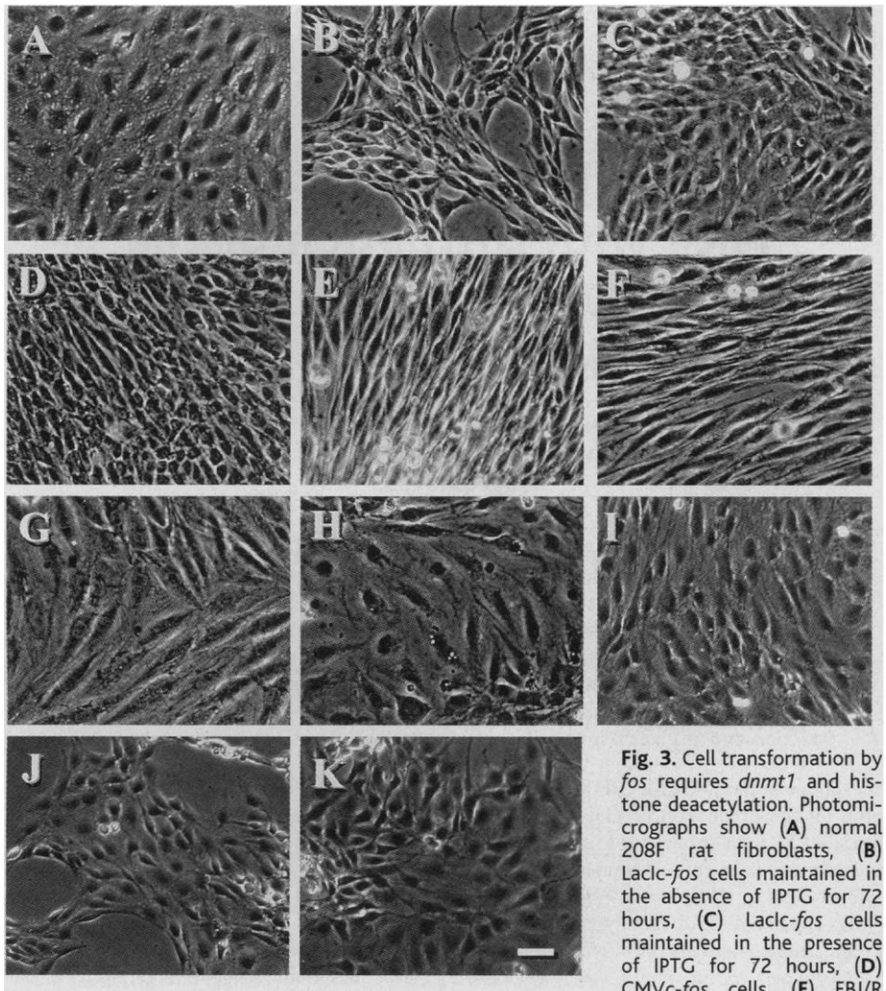

Fig. 3. Cell transformation by *fos* requires *dnmt1* and histone deacetylation. Photomicrographs show (**A**) normal 208F rat fibroblasts, (**B**) LacIc-*fos* cells maintained in the absence of IPTG for 72 hours, (**C**) LacIc-*fos* cells maintained in the presence of IPTG for 72 hours, (**D**) CMVc-*fos* cells, (**E**) FBJ/R cells, (**F**) CMV*dnmt1* cells, (**G**) CMVc-*fos* cells transfected with antisense *dnmt1*, (**H**) CMVc-*fos* cells treated with 5-aza-dC for 5 days, (**I**) CMV*dnmt1* cells treated with 80 nM TSA, (**J**) LacIc-*fos* in the absence of IPTG plus 80 nM TSA, and (**K**) FBJ/R cells treated with 120 nM TSA. Scale bar, 80 μm.

that result in transcriptional repression of nearby genes. This may occur even if the overall 5-methylcytosine content of the cell is reduced (*15*). Hypomethylation increases the frequency of rearrangement of endogenous retroviral sequences and the incidence of mutations resulting from translocations and deletions (*24*). Thus, hypomethylation has been implicated as a mutator mechanism in tumor cells, whereas hypermethylation has been linked to an epigenetic mechanism that contributes to tumorigenesis by altered gene regulation. Our results imply that, in addition to activating gene expression directly, Fos can regulate gene expression indirectly through alterations in DNA methylation and histone acetylation. DNA methylation and histone deacetylation are associated with global changes in chromatin structure and the silencing of gene expression (*15, 25*). The requirement for increased Dnmt1 (and, presumably, increased DNA methylation) explains in part the slow time course of *fos* transformation and reversion in LacIc-*fos* cells (*7*). For transformation to occur, Dnmt1 may need to increase the 5-methylcytosine content of the cell beyond a critical threshold before gene expression is affected.

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Xid-Like Immunodeficiency in Mice with Disruption of the p85α Subunit of Phosphoinositide 3-Kinase

Harumi Suzuki,* Yasuo Terauchi,* Mari Fujiwara, Shinichi Aizawa, Yoshio Yazaki, Takashi Kadowaki, Shigeo Koyasu†

Mice with a targeted gene disruption of p85α, a regulatory subunit of phosphoinositide 3-kinase, had impaired B cell development at the pro–B cell stage, reduced numbers of mature B cells and peritoneal CD5$^+$ Ly-1 B cells, reduced B cell proliferative responses, and no T cell–independent antibody production. These phenotypes are nearly identical to those of Btk$^{-/-}$ or *xid* (X-linked immunodeficiency) mice. These results provide evidence that p85α is functionally linked to the Btk pathway in antigen receptor–mediated signal transduction and is pivotal in B cell development and functions.

Phosphoinositide 3-kinase (PI3K) is responsible for the production of phosphatidylinositol-(3,4,5) trisphosphate [PtdIns $(3,4,5)P_3$] and participates in various signal transduction pathways (*1*). Heterodimer-type (Class I) PI3Ks consist of a p110 catalytic subunit and a regulatory subunit encoded by at least three distinct genes (*p85α*, *p85β*, *p55γ*) (*2*). The p85α is the most abundantly expressed regulatory isoform of PI3K, and the gene encodes two additional minor alternative splicing isoforms, p55α and p50α (*3, 4*). Binding of p85α to tyrosine-phosphorylated proteins such as IRS-1 in insulin signaling (*5*) and CD19 in B cell antigen-receptor signaling (*6*) activates PI3K activity of the p110 subunit. To elucidate precise roles of p85α in the mouse immune system in vivo, we disrupted the p85α subunit by gene targeting. Because PI3K participates in various signaling systems, disruption of the entire *p85α* gene could lead to a lethal phenotype. Thus, we