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

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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 (*p85*α, *p85*β, *p55*γ) (*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 *p85*α gene could lead to a lethal phenotype. Thus, we

disrupted the first exon of the *p85α* gene, which resulted in abrogating p85α but leaving p55α and p50α intact (*7*). Although the p85α$^{-/-}$ mice were born and grew normally under conditions free of mouse pathogens, 70 to 80% died within 10 weeks because of bacterial infection (most notably with *Corynebacterium kutcheri*) when they were housed in a conventional facility, indicating some defects in their immune system. Flow cytometric analysis of the immune system (*8*) indicated that the number of mature B cells was reduced in the p85α$^{-/-}$ mice (Fig. 1). Numbers of B220^{+}IgM^{+} mature B cells in the spleen, bone marrow, and lymph node of the p85α$^{-/-}$ mice were less than half those of control littermates (Fig. 1A). Profound reduction of the B220^{+}CD43^{-} pre–B cell population and concomitant increase of the B220^{+}CD43^{+} pro–B cell population were observed in the bone marrow of the p85α$^{-/-}$ mice (Fig. 1C), suggesting that the p85α subunit is involved in the transition from the pro-B to the pre-B stage during B cell development. In the spleen, the number of recirculating mature B cells characterized as IgM-^{lo}IgD^{hi} (*9*) was reduced in the p85α$^{-/-}$ mice (Fig. 1D). The deficient mice also had reduced numbers of IgM^{+}CD5^{+} Ly-1 B cells observed in the peritoneal cavity, compared with wild-type mice (Fig. 1E). In contrast to the defects in B cell development, no apparent defect was observed in T cell development in the thymus in either cell number or expression of CD4 or CD8 (Fig. 1B). Expression patterns of other differentiation or activation markers such as T cell receptor (TCR), CD2, CD5, CD24, and CD69 were indistinguishable between p85α$^{-/-}$ and wild-type mice (*10*). In addition, development of TCRγδ cells, natural killer cells, natural killer T cells, and intestinal intraepithelial lymphocytes were normal in the p85α$^{-/-}$ mice (*10*).

Because our targeting strategy left the expression of the p55α and p50α isoforms intact (*7*), the mutation may cause distinct effects on B and T cell development by differential expression of these alternative isoforms. The p50α isoform was expressed in T cells of both p85α$^{-/-}$ and normal mice, whereas B cells expressed only a low amount (Fig. 2). As expected, the PI3K activity of B and T cells from p85α$^{-/-}$ mice were 5 and 60% of the control mice, respectively (Fig. 2A), indicating that the mutation indeed reduced the enzymatic activity in B cells more severely than in T cells. Compared to p85α and p50α, the expression of p55α, p85β, and p55γ was negligible in T or B lymphocytes (*10*).

B cells in the p85α$^{-/-}$ mice also showed defects in their functions. Proliferative responses of p85α$^{-/-}$ B cells to bacterial lipopolysaccharide (LPS), crosslinking of antibodies to immunoglobulin M (anti-IgM), and anti-CD40 treatment (*11*) were reduced compared to those of control littermates (Fig. 3A). The proliferative responses to a combination of a phorbol ester [phorbol 12,13-dibutyrate (PDBu)] and ionomycin were indistinguishable between p85α$^{-/-}$ and normal B cells, indicating that the downstream signal transduction pathway leading to cell proliferation is intact in the absence of p85α. There was little difference in cell viability between

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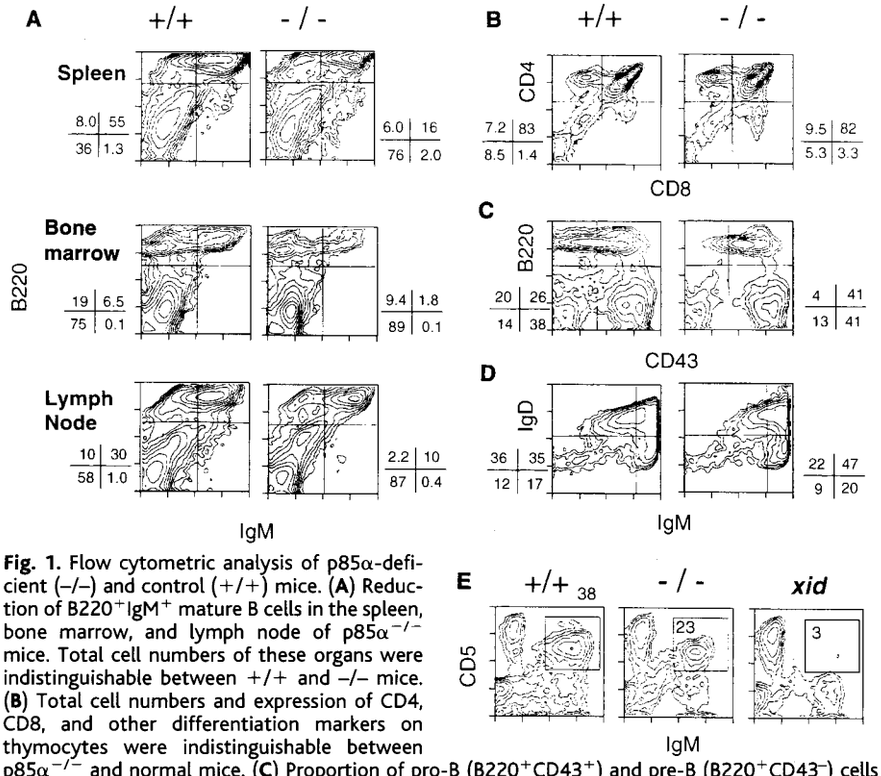

Fig. 1. Flow cytometric analysis of p85α-deficient (–/–) and control (+/+) mice. (**A**) Reduction of B220^{+}IgM^{+} mature B cells in the spleen, bone marrow, and lymph node of p85α$^{-/-}$ mice. Total cell numbers of these organs were indistinguishable between +/+ and –/– mice. (**B**) Total cell numbers and expression of CD4, CD8, and other differentiation markers on thymocytes were indistinguishable between p85α$^{-/-}$ and normal mice. (**C**) Proportion of pro-B (B220^{+}CD43^{+}) and pre-B (B220^{+}CD43^{-}) cells in bone marrow cells. Patterns of IgM^{-}-gated cells are shown. (**D**) Recirculating mature B cells (B220^{+}IgM^{lo}IgD^{hi}) in the spleen. Patterns of B220^{+}-gated cells are shown. (**E**) Proportion of CD5^{+}IgM^{+} Ly-1 B cells in the peritoneal cavity of normal and p85α$^{-/-}$ mice. Total number of peritoneal cells in p85α$^{-/-}$ mice and control littermates were 2.4 ± 0.4 × 10^{6}/mouse and 3.9 ± 0.1 × 10^{6}/mouse, respectively.

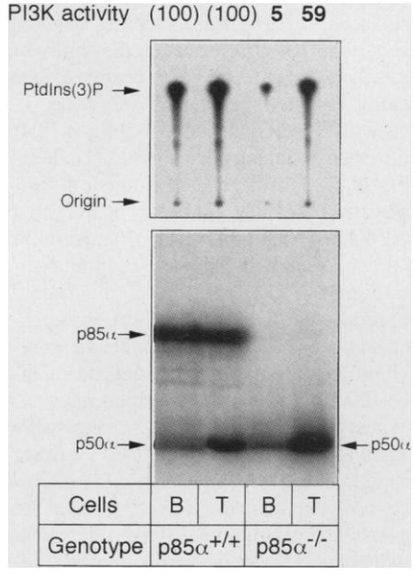

Fig. 2. Differential expression patterns of regulatory subunits of PI3K and their kinase activities in B and T lymphocytes from p85α$^{-/-}$ and normal mice. Purified B (B220^{+}) and T (CD4^{+} and CD8^{+}) cells were lysed and immunoprecipitated with the p85^{PAN} antibody (antibody to a full-length p85α–glutathione *S*-transferase fusion protein that also crossreacts with p85β and p55γ) and assayed for PI3K activity (upper panel) (*23*) or blotted with antibody to p85^{PAN} (lower panel). The identity of the p50α band was confirmed by a specific antibody [generously provided by T. Asano (*4*)] to the NH_2-terminal unique portion of p50α. PI3K activities relative to those in control mice are indicated at the top.

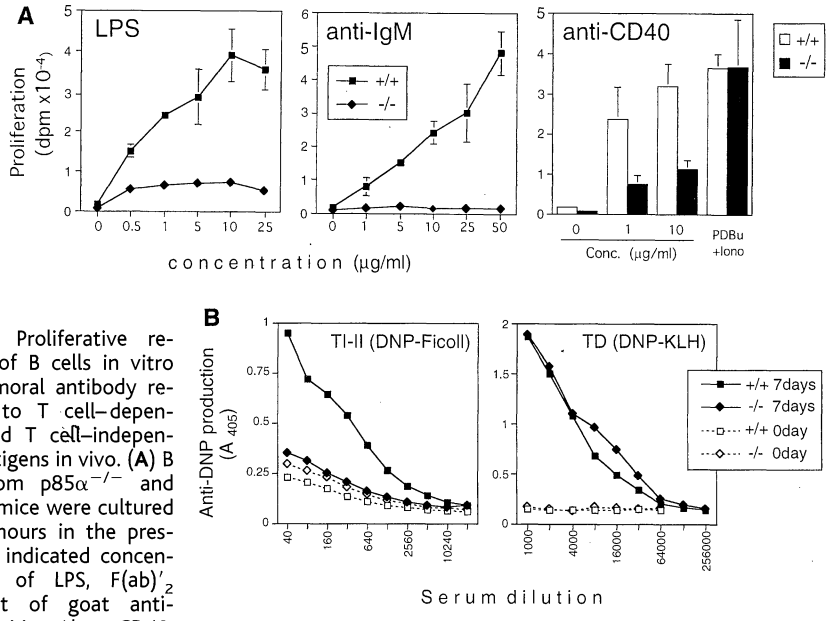

Fig. 3. Proliferative response of B cells in vitro and humoral antibody response to T cell–dependent and T cell–independent antigens in vivo. (**A**) B cells from p85α$^{-/-}$ and normal mice were cultured for 72 hours in the presence of indicated concentrations of LPS, F(ab)′$_2$ fragment of goat anti-mouse IgM, mAb to CD40, or a combination of PDBu (10 ng/ml) and ionomycin (1 μg/ml). Proliferative responses were then determined by [^{3}H]thymidine incoporation. dpm, disintegrations per minute. (**B**) Knockout and normal mice were injected with 100 μg of DNP-KLH or 10 μg of DNP-Ficoll. After 7 days, sera were collected, and DNP-specific antibody production was determined by ELISA. A_{405}, absorbance at 405 nm wavelength.

p85α$^{-/-}$ and normal B cells during B cell stimulation (*10*), further indicating that the lack of p85α had little effect on the ability of the cells to proliferate. We examined the humoral response by immunizing p85α$^{-/-}$ mice with T cell–independent antigen [2,4-dinitrophenyl–conjugated Ficoll (DNP-Ficoll)] or T cell–dependent antigen [DNP-conjugated keyhole limpet hemocyanin (DNP-KLH)], and production of antibody to DNP was examined 7 days after immunization (Fig. 3B) (*12*). The p85α$^{-/-}$ mice could not produce anti-DNP to the T cell–independent antigen (DNP-Ficoll), whereas anti-DNP production to the T cell–dependent antigen (DNP-KLH) was intact (Fig. 3B).

These phenotypes of p85α$^{-/-}$ mice were virtually identical to those of Btk$^{-/-}$ (mice lacking Bruton's tyrosine kinase) (*13*) and *xid* mice (*14*). In humans, defects in Btk are responsible for Bruton's X-linked agammaglobulinemia (XLA), a severe immunodeficiency (*15*). The pleckstrin homology (PH) domain of Btk binds to the PtdIns(3,4,5)P_3 (*16*) in the plasma membrane, and such membrane recruitment of Btk activates its kinase activity (*17*). The mutation of Btk in *xid* is in the PH domain (*18*), resulting in defective binding of its mutated PH domain to PtdIns(3,4,5)P_3. Because PI3K catalyzes the generation of PtdIns(3,4,5)P_3, it is likely that PI3K activity is critical in the activation pathway of Btk.

We observed two differences in phenotypes between our p85α$^{-/-}$ mice and Btk$^{-/-}$ or *xid* mice. First, whereas Btk$^{-/-}$ and *xid* mice completely lack peritoneal Ly-1 B cells (*10, 13, 19*), the p85α$^{-/-}$ mice have these cells, albeit in reduced numbers (Fig. 1E). Second, concentrations of natural antibodies with the IgM and IgG_3 isotypes were low in Btk$^{-/-}$ and *xid* mice (*13, 20*), whereas concentrations in the p85α$^{-/-}$ mice were normal (*10*). Because Ly-1 B cells are responsible for natural IgM and IgG_3 antibody production (*21*), incomplete blockade of Ly-1 B cell differentiation could account for the normal production of IgM and IgG_3 in the p85α$^{-/-}$ mice. The moderate phenotype of B cell deficiency in p85α$^{-/-}$ mice could be explained by residual PI3K activity (Fig. 2).

The p85α$^{-/-}$ mice still expressed minor regulatory subunits of PI3K (p85β, p55γ, p50α, and p55α) in various tissues. Targeted disruption of all isoforms derived from the *p85α* gene also caused immune-deficient phenotypes nearly identical to those of *xid* mice. However, removal of all three isoforms resulted in a nearly lethal phenotype (*22*). Thus, our mutation resulted in a comparatively mild phenotype and made it possible to analyze the function of the p85α subunit in vivo. Further analyses of the p85α$^{-/-}$ mice may uncover how PI3K functions in pathways that involve various other PH domain–containing proteins in vivo.

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11. Splenic B cells were purified using MACS magnetic cell sorting system (Miltenyi Biotec, Germany) with anti-B220–conjugated beads. Purified fraction usually contained 85 to 95% of desired cells. Purified B cells (10^5/well) were treated with the indicated concentrations of LPS (Calbiochem), F(ab)′$_2$ fragment of goat anti-mouse IgM (Cappel), monoclonal antibody (mAb) to CD40 (Pharmingen), or a combination of 10 ng/ml PDBu (Sigma) and 1 μg/ml ionomycin (Calbiochem) in 96-well plates for 72 hours. We added [^{3}H]thymidine (1 μCi/well) to the cultures during the last 16 hours, and then measured the uptake of radioactivity.
12. Mice were pre-bled and immunized intraperitoneally with 100 μg of DNP-KLH (LSL, Tokyo) in a 1:1 emulsion with Freund's complete adjuvant (Sigma) or 10 μg of DNP-Ficoll in phosphate-buffered saline at day 0. The sera were analyzed at day 7 for DNP-specific total immunoglobulin by enzyme-linked immunosorbent assay (ELISA).
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