

## Impaired Kit- but Not FcεRI-initiated Mast Cell Activation in the Absence of Phosphoinositide 3-Kinase p85α Gene Products\*

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The class I<sub>A</sub> phosphoinositide 3-kinases (PI3Ks) consist of a 110-kDa catalytic domain and a regulatory subunit encoded by the p85α, p85β, or p55γ genes. We have determined the effects of disrupting the p85α gene on the responses of mast cells stimulated by the cross-linking of Kit and FcεRI, receptors that reflect innate and adaptive responses, respectively. The absence of p85α gene products partially inhibited Kit ligand/stem cell factor-induced secretory granule exocytosis, proliferation, and phosphorylation of the serine/threonine kinase Akt. In contrast, p85α gene products were not required for FcεRI-initiated exocytosis and phosphorylation of Akt. LY294002, which inhibits all classes of PI3Ks, strongly suppressed Kit- and FcεRI-induced responses in p85α<sup>-/-</sup> mast cells, revealing the contribution of another PI3K family member(s). In contrast to B lymphocytes, mast cell proliferation was not dependent on Bruton's tyrosine kinase, a downstream effector of PI3K, revealing a distinct pathway of PI3K-dependent proliferation in mast cells. Our findings represent the first example of receptor-specific usage of different PI3K family members in a single cell type. In addition, because Kit- but not FcεRI-initiated signaling is associated with mast cell proliferation, the results provide evidence that distinct biological functions signaled by these two receptors may reflect differential usage of PI3Ks.

Mast cells (MCs)<sup>1</sup> are functionally dynamic effector cells of innate and adaptive immunity (1). Two MC surface receptors, namely, the Kit receptor (the product of the *c-kit* proto-oncogene) and the high affinity receptor for IgE (FcεRI), provide activation via innate and adaptive immune mechanisms, respectively (2–4). Kit is a receptor tyrosine kinase belonging to the colony-stimulating factor-1/platelet-derived growth factor receptor subfamily (3). Kit is encoded by the murine *White*

*Spotting* (W) locus (5, 6) and controls various cellular events during development and in adult life. Mutations at the W locus result in defects in gametogenesis, melanogenesis, and hematopoiesis (7, 8). The hematopoietic defects include macrocytic anemia (8) and the virtual absence of tissue mast cells (9). Kit is expressed on both mature MCs and their earliest progenitors (10) as well as on cells of erythroid and melanocytic lineages and on germ cells (11). Kit ligand (KL; also known as stem cell factor), is expressed in membrane-associated and soluble forms (12) by mast cells (13), fibroblasts (11), endothelial cells (14), stromal cells (15), keratinocytes (16), neuroblastoma cells (17), and tumor cell lines (18). Although KL represents a major growth and differentiation factor for both murine and human MCs (19, 20), it also promotes Kit-dependent MC mediator release (21–23), as well as enhances the release of MC mediators via IgE-dependent mechanisms (22, 24). FcεRI belongs to the antigen receptor superfamily (4). Rodent FcεRI is a tetrameric receptor consisting of an α chain, β chain, and a dimer of disulfide-linked γ chains, whereas human FcεRI exists both as trimeric (αγ<sub>2</sub>) and tetrameric (αβγ<sub>2</sub>) structures (4, 25). The α chain binds IgE, the γ chains are essential for signal transduction, and the β chain acts as an amplifier of signaling (26). Rodent FcεRI is strictly expressed on mast cells, basophils, and non-B, non-T cells, whereas the expression of human FcεRI also includes dendritic cells, eosinophils, Langerhans cells, platelets, and monocytes (4, 27–31). FcεRI plays a critical role in allergic reactions because it is the major surface receptor through which MCs direct immunologically specific secretory effects, such as the release of preformed cytoplasmic granule-associated mediators and the generation and release of lipid mediators and cytokines (32).

PI3Ks are a family of lipid kinases that phosphorylate phosphatidylinositol (PtdIns), PtdIns-4-phosphate, or PtdIns-4,5-bisphosphate at the 3'-position of the inositol ring to generate PtdIns-3-phosphate, PtdIns-3,4-bisphosphate, and PtdIns-3,4,5-trisphosphate, respectively (33, 34). In response to a variety of signals, PI3Ks are involved in the regulation of many cellular functions ranging from cytoskeletal reorganization, secretion, vesicular sorting, cell migration, protein synthesis, and cell survival (33, 34). PtdIns-3,4-bisphosphate and PtdIns-3,4,5-trisphosphate interact directly with the pleckstrin homology domains of intracellular proteins such as Btk, the serine/threonine kinase Akt, phosphoinositide-dependent kinase-1, and phospholipase C-γ, thereby targeting these molecules to the plasma membrane and facilitating their activation for downstream signaling (35–41). Nine members of the PI3K family have been isolated from mammalian cells, and they are grouped into three classes (33, 42). Heterodimeric, class I<sub>A</sub> PI3Ks consist of a 110-kDa catalytic subunit (p110α, β, or δ)

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<sup>1</sup> The abbreviations used are: MC, mast cell; KL, Kit ligand; FcεRI, the high affinity receptor for IgE; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; Btk, Bruton's tyrosine kinase; IL, interleukin; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate; BMDC, bone marrow-derived mast cell; FLDC, fetal liver-derived mast cell; MAR, F(ab')<sub>2</sub> mouse anti-rat IgG (heavy and light chain reactive); Ab, antibody; Xid, X-linked immunodeficiency.

and a regulatory subunit (p85 $\alpha$ , p85 $\beta$ , or p55 $\gamma$ ) combined in an apparently nonpreferential manner (42). The gene encoding p85 $\alpha$  produces two additional isoforms, p55 $\alpha$  and p50 $\alpha$ , by alternate splicing or promoter usage (43). The regulatory subunits possess no enzymatic activity but are composed of several domains capable of interacting with other signaling proteins. The Src homology-2 domains of p85 bind selectively to phosphotyrosyl residues within a *p*-Tyr-Xaa-Xaa-Met sequence motif, where Xaa is any amino acid (44). Synthetic peptides containing tandem *p*-Tyr-Met-Xaa-Met motifs bind to p85 proteins with high affinity and increase the catalytic activity of the associated p110 subunits 2- to 3-fold (45). Thus, p85 proteins regulate the activities and subcellular locations of class I<sub>A</sub> PI3Ks.

The signaling pathways initiated by the stimulation of mast cells through the Kit tyrosine kinase and Fc $\epsilon$ RI, which lacks intrinsic kinase activity, include kinase activation, receptor phosphorylation and association with various intracellular signaling molecules, and activation of PI3Ks, but these events occur in a different manner for Kit and Fc $\epsilon$ RI. The dimerization of Kit by KL causes receptor autophosphorylation (46) leading to the direct binding of one or more of the p85 subunits of the class I<sub>A</sub> PI3Ks to Tyr-719 (47, 48) and PI3K activation (49). Elimination of the PI3K-binding site by substitution of Tyr-719 with phenylalanine reduces the rate of Kit-mediated proliferation (48) and abolishes Kit-mediated potentiation of Fc $\epsilon$ RI-induced secretion in mast cells (50). Because Fc $\epsilon$ RI does not have intrinsic kinase activity, signaling by aggregated Fc $\epsilon$ RI depends on the Src family kinase Lyn, which is associated with the  $\beta$ -chain, to phosphorylate tyrosines on the  $\beta$  and  $\gamma$  chains within a sequence known as the immunoreceptor tyrosine-based activation motif (51). The phosphorylated tyrosines target Syk to the plasma membrane, where it is phosphorylated by Lyn, resulting in Syk activation (52, 53). Activated Syk then phosphorylates a number of substrates and ultimately, PI3K is activated (54, 55). The exact nature of the interactions of PI3K within the Fc $\epsilon$ RI signaling cascade is unclear. Nonetheless, PI3K activity is required for maximal Fc $\epsilon$ RI-induced calcium influx, degranulation, c-Jun amino-terminal kinase activation, and cytokine production as assessed with pharmacological inhibitors of PI3K such as wortmannin and LY294002 (56, 57). However, these broad spectrum PI3K inhibitors inhibit all three classes of PI3Ks. Therefore, it is not known whether Kit or Fc $\epsilon$ RI use the same or different class(es) of PI3K.

To address this issue, we have compared Kit- and Fc $\epsilon$ RI-induced activation events in mast cells derived from p85 $\alpha$  wild-type (+/+) versus homozygous-deficient (-/-) fetal livers. The results demonstrate that p85 $\alpha$  gene products are not required for Fc $\epsilon$ RI-mediated mast cell exocytosis and phosphorylation of Akt but are essential for maximal Kit-mediated exocytosis, proliferation, and Akt phosphorylation. Our findings represent the first demonstration that Kit and Fc $\epsilon$ RI utilize distinct members of the PI3K family.

#### EXPERIMENTAL PROCEDURES

**Reagents**—Mouse recombinant KL and IL-3 were expressed by the infection of Sf9 insect cells with recombinant baculovirus (58). Rat mAb 2.4G2 anti-mouse Fc $\gamma$ RII/III, mouse IgE mAb IgE-3 anti-trinitrophenyl (TNP), FITC-rat mAb R35-72 anti-mouse IgE, FITC-rat mAb 2B8 anti-mouse Kit, and FITC-rat IgG<sub>2b</sub> were purchased from Pharmingen. The rabbit polyclonal Abs for Akt and *p*-Akt were obtained from New England Biolabs. Mouse mAb anti-p85 $\alpha$  and rabbit polyclonal anti-pan p85 (Upstate Biotechnology Inc.), rabbit polyclonal anti-p85 $\beta$  (59), p110 $\alpha$  (Santa Cruz), p110 $\beta$  (Santa Cruz), p110 $\delta$  (gift from Bart Vanhaesebroeck), p55 $\gamma$  (gift of Ivan Gout), rat IgE mAb LO-DNA-30 anti-DNP (Serotec), F(ab')<sub>2</sub> mouse anti-rat IgG (heavy and light chain reactive) (MAR) (Jackson ImmunoResearch Laboratories), horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) and goat anti-rabbit IgG (New England Biolabs), and anti-phosphotyrosine mAb

4G10 (Upstate Biotechnology Inc.) were obtained as noted.

**Mast Cell Cultures**—Fetal livers from 15.5-day-old embryos resulting from the mating of p85 $\alpha$  +/- mice (129/Sv  $\times$  C57BL/6) were dispersed mechanically. The genotypes of the fetuses were determined by polymerase chain reaction analysis as described previously (59). Fetal liver cells were cultured at  $5 \times 10^5$  cells/ml in medium (RPMI 1640 medium containing 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10% fetal calf serum) containing 50% WEHI-3 cell-conditioned medium. Nonadherent cells were passed weekly. Bone marrow cells from the femurs of CBA/J and CBA/CaHN-xid/J mice (Jackson Labs) were cultured identically.

**Flow Cytometric Analyses**—To measure Fc $\epsilon$ RI expression, IgG-Fc receptors were first blocked by incubating cells with rat mAb 2.G2 for 15 min, followed by incubation for 50 min on ice with or without mouse mAb IgE anti-DNP. The cells were washed and stained with FITC rat anti-mouse IgE for 25 min on ice. Kit expression was measured by incubating the cells with FITC rat mAb 2B8 anti-mouse Kit or with the isotype-matched negative control, FITC rat IgG<sub>2b</sub>, for 30 min on ice. Cells were analyzed on a Becton Dickinson FACSort with logarithmic fluorescence amplification.

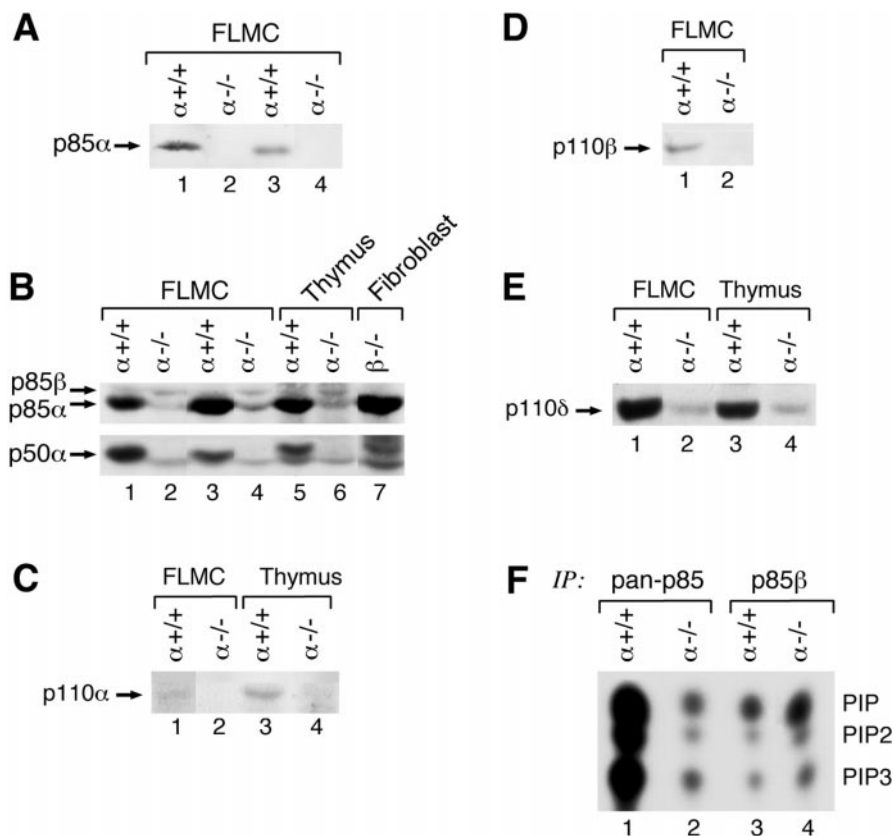
**Mast Cell Activation for Exocytosis**—For KL-mediated activation, cells were incubated at  $1 \times 10^7$  cells/ml in medium alone or containing the indicated concentrations of KL. For IgE-induced activation, cells were sensitized at  $1 \times 10^7$  cells/ml in medium alone or containing the indicated concentrations of rat mAb IgE anti-DNP for 1 h on ice. The cells were washed by centrifugation, and pellets were resuspended on ice in their original volume in medium with 25  $\mu$ g/ml MAR. With both agonists, the reactions were stopped by centrifugation after incubation for 20 min at 37 °C. The supernatants were saved, and the pellets were resuspended in their original volume with medium and lysed by three cycles of freezing in an alcohol/dry ice bath and thawing at 37 °C. For the  $\beta$ -hexaminidase assay, aliquots (10  $\mu$ l) of supernatants and cell lysates were incubated for 30 min at 37 °C with 80  $\mu$ l of substrate solution (1.3 mg/ml *p*-nitrophenyl- $\beta$ -D-2-acetamido-2-deoxyglucopyranoside in 0.1 M citrate buffer, pH 4.5). The reactions were stopped by the addition of 200  $\mu$ l of 0.2 M glycine (pH 10.7) and OD was read at 405 nm in an enzyme-linked immunosorbent assay reader.

**Viable Cell Number and Thymidine Incorporation Analyses**—Cells were washed and incubated at 37 °C at  $1 \times 10^6$ /ml in medium alone or containing the indicated concentrations of KL or IL-3, with or without 10  $\mu$ M LY294002 (Calbiochem). After 1, 2, and 3 days, cells were stained with trypan blue and counted with a hemacytometer. For measurement of DNA synthesis, cells were starved for 2 h in medium, and  $5 \times 10^4$  cells were seeded in 100  $\mu$ l in triplicate in 96-well plates and maintained in medium alone or with the indicated concentrations of cytokines, with or without LY294002. After 24 h, 0.5  $\mu$ Ci of [<sup>3</sup>H]thymidine (2 Ci/mmol) was added to each well. After an additional 12 h, cells were harvested onto a filter, and the filter-bound radioactivity was measured in a liquid scintillation counter.

**Cell Cycle and Apoptosis Analyses**—For cell cycle analysis,  $1 \times 10^6$  cells were washed once and resuspended in 1 ml of cold phosphate-buffered saline, 5 mM EDTA. Cells were fixed by slowly adding 1 ml of 100% ethanol while vortexing and incubated for 30 min at room temperature. Cells were pelleted, resuspended in 0.5 ml of phosphate-buffered saline, 5 mM EDTA containing 40  $\mu$ g/ml RNase A (Ambion), and incubated 30 min at room temperature. Propidium iodide (Sigma) (0.5 ml of a 100  $\mu$ g/ml solution in phosphate-buffered saline, 5 mM EDTA) was added, and the cells were incubated at 4 °C until samples were analyzed for cell cycle and apoptosis by FACS using Modfit software. Detection of apoptosis by FITC-Annexin V staining was performed according to the manufacturer's instructions (Pharmingen).

**Immunoblotting**—Cells were starved for 20 h in medium, then incubated at  $1 \times 10^7$  cells/ml in Hanks' Balanced Salt Solution containing 1 mM each of CaCl<sub>2</sub> and MgCl<sub>2</sub> (CM buffer) for 30 min on ice, and stimulated with KL (1/100) or IgE (5  $\mu$ g/ml) + MAR (25  $\mu$ g/ml) in CM buffer as described above. The cells were lysed at  $5 \times 10^7$  cells/ml with 1% Nonidet P-40 extraction buffer (60). For Western blotting, 20  $\mu$ l of lysate ( $1 \times 10^6$  cell equivalents) was mixed with 10  $\mu$ l of 3 $\times$  sample buffer containing 15%  $\beta$ -mercaptoethanol and loaded on precast Tris-glycine gels (Novex). Proteins were transferred to polyvinylidene difluoride membranes (Millipore) by electroblotting. For p85 and p110 immunoblotting, 100  $\mu$ g of protein was loaded on 6 or 7% gels and transferred to nitrocellulose. Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 2 h and incubated with primary antibodies in blocking buffer overnight. Membranes were then washed three times, incubated at room temperature with appropriate secondary antibodies for 1 h, and washed three times. Immunoreactive proteins were visualized with chemiluminescence.

**FIG. 1. Expression levels of the p85 regulatory subunits and p110 catalytic subunits of the class I<sub>A</sub> PI3Ks and PI3K enzymatic activity in p85 $\alpha$  +/+ and -/- FLMC.** Whole cell lysates (100  $\mu$ g of protein) were immunoblotted with (A) p85 $\alpha$ -specific mAb, (B) pan-p85 Ab, (C) p110 $\alpha$  Ab, (D) p110 $\beta$  Ab, and (E) p110 $\delta$  Ab. In A and B, lanes 1 and 2 and lanes 3 and 4 contain samples from different lots of FLMC. F, cell lysates ( $1 \times 10^7$  cells) were immunoprecipitated with either pan-p85 Ab (lanes 1 and 2) or p85 $\beta$ -specific Ab (lanes 3 and 4). Kinase reactions were performed for 5 min and 15 min with the pan-p85 and p85 $\beta$  immunoprecipitates, respectively. In lane 1, only 1/5th of the kinase reaction product was loaded on the TLC plate. PIP, PtdIns-3-phosphate; PIP2, PtdIns-3,4-bisphosphate; PIP3, PtdIns-3,4,5-trisphosphate.



**PI3-kinase Assay**—Cells ( $1 \times 10^7$ ) were starved, incubated in CM buffer as described for immunoblotting, and subjected to immunoprecipitation with polyclonal anti-pan-p85 antibody, polyclonal anti-p85 $\beta$  antibody, or anti-phosphotyrosine antibody 4G10. Immune complex kinase assays were performed with a substrate mixture of PtdIns, PtdIns-4-phosphate, and PtdIns-4,5-bisphosphate in the presence of phosphatidylserine carrier as described (61). The lipid products were separated by thin-layer chromatography, and radioactivity in the PtdIns-3,4,5-trisphosphate spot was quantitated with a Molecular Imager (Bio-Rad).

## RESULTS

**Products of the p85 $\alpha$  Gene Are Not Required to Generate Mast Cells from Fetal Liver in the Presence of IL-3**—Because mice homozygous for disruption of all three isoforms of p85 $\alpha$  gene products die perinatally (59), we grew mast cells from the fetal livers of 15.5-day-old p85 $\alpha$  +/+ and -/- embryos in medium containing IL-3. After 3–4 weeks, cultures from both genotypes consisted of similar numbers of >99% fetal liver-derived mast cells (FLMC), as determined by metachromatic staining with toluidine blue. In a single experiment, mast cells grew comparably from the bone marrow of a p85 $\alpha$  -/- mouse that survived for several weeks, compared with cells grown from the bone marrow of a p85 $\alpha$  +/+ littermate.

The absence of p85 $\alpha$  protein in p85 $\alpha$  -/- FLMC was ascertained with a mAb directed specifically to p85 $\alpha$  (Fig. 1A). Immunoblot analysis of FLMC, thymocyte, and fibroblast lysates with a pan-p85 Ab that recognizes the p85 $\alpha$ , p55 $\alpha$ , and p50 $\alpha$  splice variants as well as p85 $\beta$  confirmed the loss of p85 $\alpha$  and p50 $\alpha$  protein in p85 $\alpha$  -/- FLMC and thymocytes and revealed that, unlike thymocytes, FLMC did not express p55 $\alpha$  detectably (Fig. 1B). Expression of the p85 $\beta$  gene product, identified by its absence in p85 $\beta$  -/- fibroblasts, appeared to be up-regulated in FLMC lacking p85 $\alpha$ , as reported for other cell types (59). The expression of the PI3K p55 $\gamma$  gene product was not detected by immunoblotting in FLMC of either genotype (data not shown). Hence, p85 $\alpha$  +/+ FLMC expressed p85 $\alpha$ ,

p50 $\alpha$ , and p85 $\beta$  of the class I<sub>A</sub> PI3K adapter subunits. Expression of the class I<sub>A</sub> PI3K catalytic subunits p110 $\alpha$ , p110 $\beta$ , and p110 $\delta$  was reduced in p85 $\alpha$  -/- FLMC (Figs. 1, C–E, respectively), indicating that p85 $\alpha$  regulatory subunits are required to stabilize expression of not only p110 $\alpha$  (62), but all three p110 isoforms. The pan-p85 antibody immunoprecipitated a small amount of PI3K activity in p85 $\alpha$  -/- FLMC ( $2.8 \pm 1.3\%$  of the amount from p85 $\alpha$  +/+,  $n = 3$ ; Fig. 1F), whereas the amount of PI3K activity associated with p85 $\beta$  immunoprecipitates in p85 $\alpha$  -/- FLMC was 184% of the amount in p85 $\alpha$  +/+ FLMC in one experiment (Fig. 1F), consistent with the changes in immunoreactivity. Hence, p85 $\alpha$  gene products and a substantial portion of class I<sub>A</sub> PI3K activity are not required for the growth and development of mast cells from fetal liver in the presence of IL-3.

**p85 $\alpha$  Deficiency Results in Reduced Secretory Granule Exocytosis by Mast Cells in Response to KL but Not IgE Cross-linking**—As assessed by flow cytometry, FLMC of both genotypes expressed comparable levels of Kit and Fc $\epsilon$ RI (Fig. 2). FLMC derived from p85 $\alpha$  +/+ and -/- mice were incubated with several concentrations of KL for 20 min at 37 °C. Separate samples were incubated with rat IgE for 1 h and washed, and then the bound IgE was cross-linked with MAR for 20 min at 37 °C. Stimulation with KL resulted in a dose-dependent release of  $\beta$ -hexosaminidase from FLMC with both genotypes (Fig. 3A). However, there was an approximately 50% reduction in  $\beta$ -hexosaminidase release from p85 $\alpha$  -/- cells at each concentration of KL. In contrast, there was no significant difference between the ability of mast cells of each genotype to degranulate upon IgE cross-linking at the IgE concentrations tested ( $p > 0.5$ ; one way ANOVA (analysis of variance)), which each provided maximal activation (Fig. 3B). In one experiment, a lower concentration of IgE (1.25  $\mu$ g/ml) elicited 52% and 41%  $\beta$ -hexosaminidase release from p85 $\alpha$  +/+ and p85 $\alpha$  -/- cells, respectively. Pretreatment of cells with the PI3K inhibitor

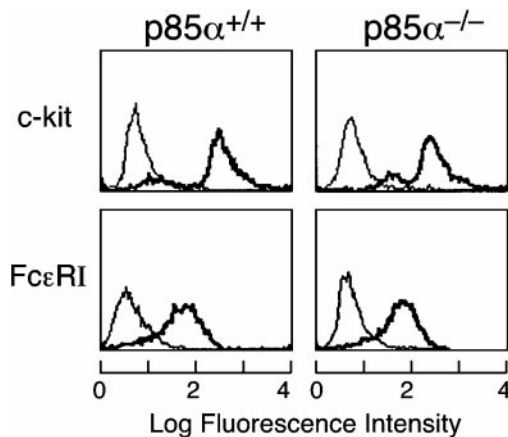


FIG. 2. FACS analysis of Kit and Fc $\epsilon$ RI expression on p85 $\alpha$   $+/+$  and  $-/-$  FLMC. Fc $\epsilon$ RI expression was measured by first blocking IgG-Fc receptors with rat mAb 2.4G2, followed by incubation with (bold line) and without (thin line) mouse mAb IgE anti-DNP. The cells were washed and stained with FITC rat anti-mouse IgE. Kit expression was measured by incubating the cells with FITC rat mAb 2B8 anti-mouse Kit (bold line) or with the isotype-matched negative control, FITC-rat IgG<sub>2b</sub> (thin line).

LY294002, which inhibits all three classes of PI3Ks, inhibited the release of  $\beta$ -hexosaminidase in both genotypes to a similar extent ( $\sim$ 75%) for both agonists, indicating that a PI3K not requiring a p85 $\alpha$  gene product is critical for Fc $\epsilon$ RI-induced exocytosis and also contributes partially to Kit-mediated exocytosis.

**KL-dependent Proliferation Is Reduced in Mast Cells Deficient in p85 $\alpha$  Gene Products**—FLMC generated in medium containing IL-3 were switched to medium alone or containing either recombinant IL-3, KL, or KL + LY294002. Cell viability assessments with trypan blue exclusion indicated that the number of p85 $\alpha$   $+/+$  FLMC increased 60% over 3 days in response to IL-3 and remained relatively constant during the same period in response to KL (Fig. 4A). In contrast, the viable cell numbers decreased by  $\sim$ 60 and 95% in KL + LY294002 or medium alone, respectively. Compared with p85 $\alpha$   $+/+$  cells, similar numbers of viable FLMC were obtained when p85 $\alpha$   $-/-$  cells were treated with IL-3 in medium. However, the number of p85 $\alpha$   $-/-$  FLMC decreased by 70% after 3 days of culture in KL. This decrease is comparable to the 60 and 80% decreases after culture of p85 $\alpha$   $+/+$  and  $-/-$  FLMC, respectively, in KL + LY294002. When p85 $\alpha$   $+/+$  and  $-/-$  FLMC were cultured for 3 days in the presence of IL-3 + LY294002, the numbers of viable p85 $\alpha$   $+/+$  and  $-/-$  FLMC were both decreased by  $\sim$ 50% (data not shown).

To investigate whether the decrease in KL-mediated cell viability in p85 $\alpha$   $-/-$  FLMC could be attributed to a lack of proliferation, a block in cell cycle progression, or increased cell death, we performed DNA synthesis, cell cycle, and apoptosis assays. As assessed by the incorporation of tritiated thymidine, DNA synthesis in the presence of IL-3 for 36 h was the same in cells derived from both genotypes (Fig. 4B). However, p85 $\alpha$   $-/-$  FLMC cultured in KL for the same period had a drastically impaired ability to synthesize DNA compared with p85 $\alpha$   $+/+$  FLMC (Fig. 4B). Furthermore, DNA synthesis was essentially completely inhibited in both genotypes when LY294002 was present together with KL. Cell cycle analysis using propidium iodide supported these results (Fig. 4C). There were no appreciable differences between p85 $\alpha$   $+/+$  and  $-/-$  FLMC in the percentages of cells in the G<sub>0</sub>/G<sub>1</sub>, G<sub>2</sub>/M, and S phases of cell cycle after 24 h of culture with IL-3, KL + LY294002, or medium alone. However, the percentage of p85 $\alpha$   $+/+$  FLMC in S phase in the presence of KL was approximately 6-fold greater

than p85 $\alpha$   $-/-$  FLMC. Neither annexin V nor propidium iodide analyses showed increases in apoptotic cells when p85 $\alpha$   $-/-$  FLMC were treated with KL, compared with p85 $\alpha$   $+/+$  FLMC (data not shown).

**KL-dependent Proliferation Is Normal in Mast Cells Derived from X-linked Immunodeficiency (Xid) Mice**—An Arg to Cys mutation at position 28 in the pleckstrin homology domain of Btk (63) causes Xid in mice (64, 65) and eliminates the selective recruitment of Btk to the plasma membrane by PtdIns-3,4,5-trisphosphate (35, 36). The similar impairments in *in vivo* B cell development and *in vitro* proliferation in p85 $\alpha$   $-/-$  and Xid mice (59, 66) provided a genetic link between PI3K and Btk in B cell signaling. Hence, we examined the proliferative responses of bone marrow-derived mast cells (BMMC) from Xid and control CBA/J mice in the experimental conditions described above. Xid mast cells have been shown to be deficient in certain Fc $\epsilon$ RI-dependent responses (67). Surprisingly, cell viability assessments with trypan blue exclusion indicated that there were no appreciable differences in the responses of BMMC from Xid and CBA/J mice over 3 days to IL-3 or KL, respectively (Fig. 5A). Moreover, the ability of Xid BMMC to synthesize DNA in the presence of KL was intact (Fig. 5B). Cell cycle analysis also demonstrated comparable progression of Xid BMMC through S phase upon KL stimulation, relative to CBA/J BMMC (Fig. 5C). These results indicate that the PI3K-dependent proliferative response to KL in BMMC does not require Btk.

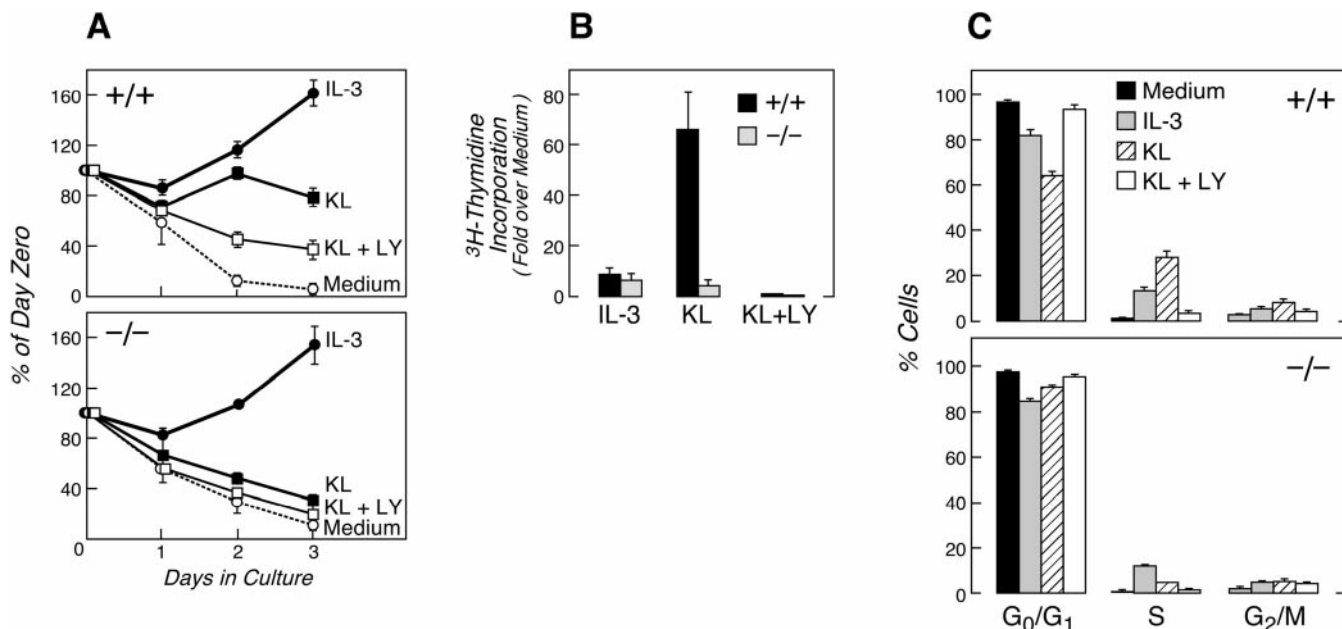
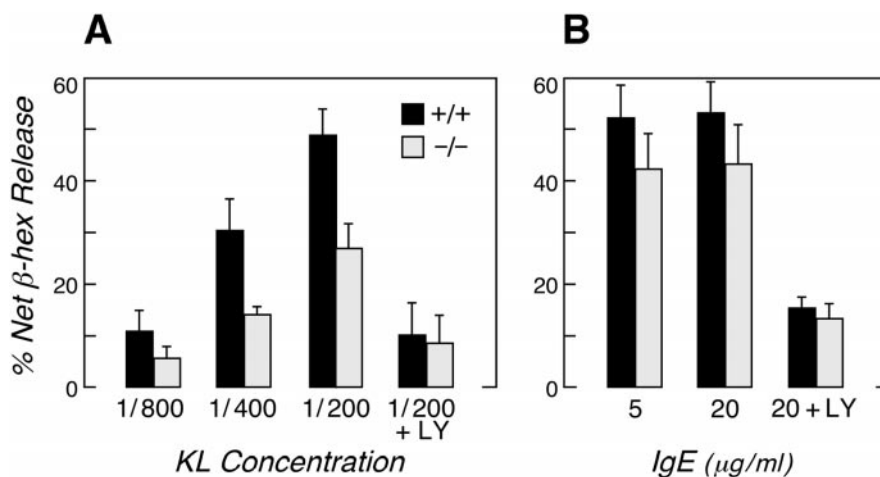
**KL-dependent PI3K Activity Is Diminished in p85 $\alpha$ -deficient Mast Cells**—To determine the effect of p85 $\alpha$  deficiency on Kit- and Fc $\epsilon$ RI-induced PI3K activity, we measured PI3K activity immunoprecipitated with an anti-phosphotyrosine antibody from p85 $\alpha$   $+/+$  and  $-/-$  FLMC activated via Kit or Fc $\epsilon$ RI. Treatment with KL caused an increase in phosphotyrosine-associated PI3K activity in both p85 $\alpha$   $+/+$  and  $-/-$  FLMC. However, the activity was almost 4-fold greater in the p85 $\alpha$   $+/+$  cells (Fig. 6, A and B). The kinase activity was mediated by class I<sub>A</sub> PI3Ks as judged by the phosphorylation of all three substrates and inhibition by a low concentration (10  $\mu$ M) of LY294002 (42). Treatment with IgE + MAR did not induce significant changes in phosphotyrosine-associated PI3K activity in p85 $\alpha$   $+/+$  and  $-/-$  FLMC (data not shown).

**KL-dependent Akt Activation Is Diminished in p85 $\alpha$ -deficient Mast Cells But Not in Xid Mast Cells**—The Akt proto-oncogene encodes a PI3K-dependent serine/threonine kinase (68), and Akt phosphorylation is commonly used as an *in vivo* indicator of PI3K activity (69, 70). We examined the expression and phosphorylation of Akt in p85 $\alpha$   $+/+$  and  $-/-$  FLMC and in CBA/J and Xid BMMC by immunoblotting (Fig. 7). Akt was constitutively expressed in all populations. Akt became rapidly phosphorylated upon KL stimulation or IgE cross-linking in p85 $\alpha$   $+/+$  mast cells (Fig. 7, A and B). However, in p85 $\alpha$   $-/-$  mast cells, KL triggered a smaller increase in Akt phosphorylation at each time point examined (Fig. 7A). In contrast, the phosphorylation of Akt was undiminished after IgE cross-linking and appeared to be increased in  $-/-$  compared with  $+/+$  FLMC (Fig. 7B). Pretreatment of p85 $\alpha$   $+/+$  and  $-/-$  FLMC with LY294002 followed by stimulation with either agonist reduced the phosphorylation of Akt to basal levels (data not shown). In contrast with p85 $\alpha$   $-/-$  FLMC, the phosphorylation of Akt was essentially the same in response to KL in Xid and CBA/J BMMC (Fig. 7C).

## DISCUSSION

We have shown in this study that a product(s) of the PI3K p85 $\alpha$  gene is critical for maximal Kit-mediated exocytosis, proliferation, and phosphorylation of Akt in mast cells, but is dispensable for Fc $\epsilon$ RI-induced exocytosis and phosphorylation

**FIG. 3. Effects of p85 $\alpha$  gene deficiency on Kit- and Fc $\epsilon$ RI-mediated exocytosis.** p85 $\alpha$   $+/+$  and  $-/-$  FLMC at  $1 \times 10^7$ /ml were stimulated with the indicated dilutions of KL (A) or sensitized with the indicated amounts of rat IgE and activated by adding MAR (25  $\mu$ g/ml) to cross-link the IgE (B). Cells were also preincubated with 10  $\mu$ M LY294002 for 15 min at 37  $^{\circ}$ C and then stimulated with the highest dose of either agonist. The data are expressed as the net percentage of  $\beta$ -hexosaminidase released (mean  $\pm$  S.E.;  $n = 3-4$ ).



**FIG. 4. Effects of p85 $\alpha$  gene deficiency on cell proliferation.** A, p85 $\alpha$   $+/+$  and  $-/-$  FLMC at  $1 \times 10^6$ /ml were incubated in medium alone or supplemented with IL-3 (1/200), KL (1/400), or KL (1/400) + LY294002 (10  $\mu$ M). Viable cell numbers were determined with trypan blue on days 0, 1, 2, and 3. B, cells were starved for 2 h in medium, seeded at  $5 \times 10^4$  cells/well in a 96-well plate, and then incubated in medium alone or supplemented as in A for 36 h. During the last 12 h of incubation, 0.5  $\mu$ Ci of [ $^3$ H]thymidine was added per well. Data are presented as the ratios of cpm with the different treatments to cpm with medium alone in the respective cultures. C, p85 $\alpha$   $+/+$  and  $-/-$  FLMC were cultured as in A for 24 h and then subjected to cell cycle analysis with propidium iodide staining. All data (A-C) are expressed as mean  $\pm$  S.E.,  $n = 3$ .

of Akt, as well as for the development and proliferation of mast cells from fetal liver and bone marrow progenitors in a source of IL-3. The studies also demonstrate that Btk plays a less critical role in PI3K signaling for cell proliferation in mast cells than in B cells (59, 66).

The growth of FLMC from p85 $\alpha$   $-/-$  embryos in IL-3 containing medium was normal in terms of cell numbers, metaphase staining, and cell surface expression of Kit and Fc $\epsilon$ RI (Fig. 2). FLMC derived from p85 $\alpha$   $-/-$  mice exhibited a complete loss of the two p85 $\alpha$  gene products that were expressed in p85 $\alpha$   $+/+$  FLMC, namely, the p85 $\alpha$  and p50 $\alpha$  proteins (Fig. 1). In contrast, expression of the p85 $\beta$  gene product was augmented in p85 $\alpha$   $-/-$  FLMC. In addition, there was a dramatic decrease in the expression of not only the p110 $\alpha$  PI3K catalytic subunit, as previously shown in p85 $\alpha$   $-/-$  lymphocytes (59), but also the p110 $\beta$  and p110 $\delta$  subunits (Fig. 1), demonstrating that the expression levels of all three catalytic subunits are regulated by the presence of p85 $\alpha$  gene products. In agreement with the decrease in expression of the regulatory and catalytic units, p85 $\alpha$   $-/-$  FLMC had approximately 3% of

the class I $_A$  PI3K activity of p85 $\alpha$   $+/+$  FLMC, as assessed by *in vitro* PI3K assay of pan-p85 immunoprecipitates (Fig. 1).

The absence of p85 $\alpha$  gene products resulted in  $\sim$ 50% inhibition of Kit-induced exocytosis of  $\beta$ -hexosaminidase from FLMC, but there was no appreciable attenuation of Fc $\epsilon$ RI-mediated exocytosis (Fig. 3). However, the addition of the broad-spectrum PI3K inhibitor LY294002 strongly suppressed exocytosis in response to the cross-linking of either receptor in both p85 $\alpha$   $+/+$  and p85 $\alpha$   $-/-$  FLMC (Fig. 3), which has been observed previously (in some cases using Wortmannin) for Fc $\epsilon$ RI-induced activation of the rat basophilic leukemia cell line (54) and BMMC (48, 56, 71, 72). Hence, our studies establish that there is a pool of LY294002-sensitive, p85 $\alpha$ -independent PI3K molecules that are essential for Fc $\epsilon$ RI-induced exocytosis. These molecules do not appear to be the other class I $_A$  regulatory subunits, namely p55 $\gamma$ , which was not detected in FLMC, or p85 $\beta$ , which is not necessary for IgE-dependent exocytosis, as determined with BMMC grown from p85 $\beta$   $-/-$  mice in a source of IL-3 (data not shown). It remains possible that the increase in p85 $\beta$  expression in p85 $\alpha$   $-/-$  FLMC may mediate a

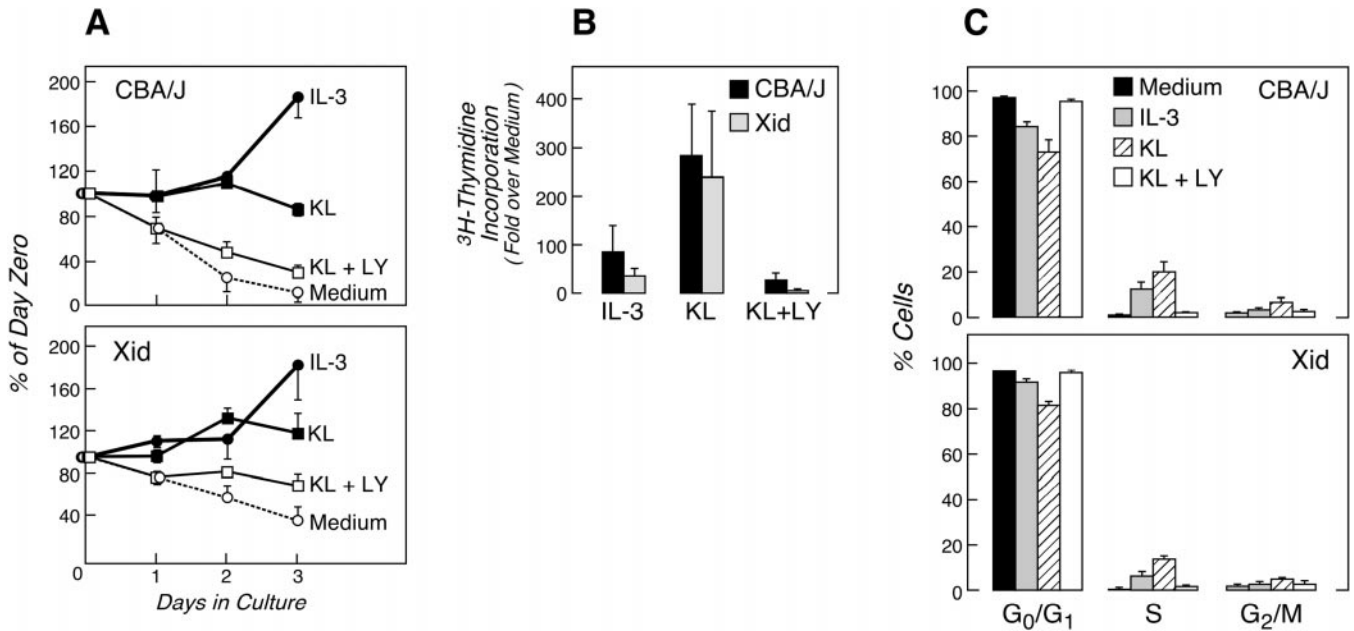


FIG. 5. Effects of Xid mutation on KL-dependent proliferation in mast cells. Xid and CBA/J control BMMC were treated as in Fig. 4 and assessed for viable cell number (A), [<sup>3</sup>H]thymidine incorporation (B), and cell cycle analysis (C). All data (A–C) are expressed as mean  $\pm$  S.E.,  $n = 3$ .

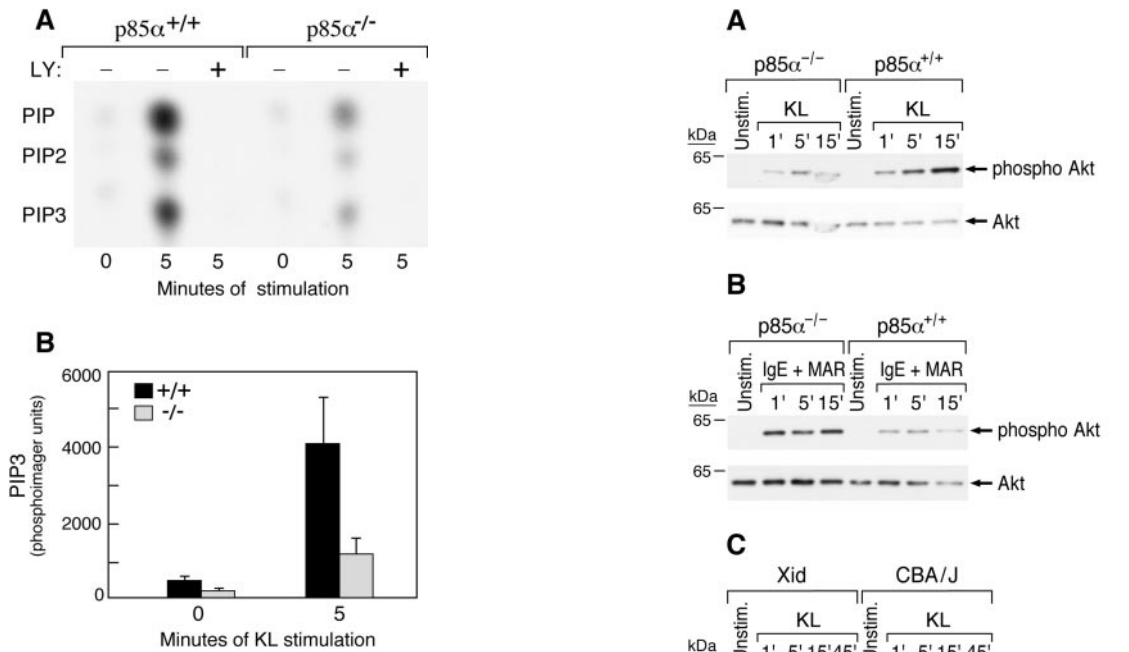


FIG. 6. Effects of p85 $\alpha$  gene deficiency on phosphotyrosine-associated PI3K activity. A, cells were starved as described under “Experimental Procedures” and stimulated as in Fig. 3 with KL (1/100) for 5 min. Cell lysates ( $1 \times 10^7$  cells) were immunoprecipitated with anti-phosphotyrosine mAb 4G10, and kinase reactions were performed for 5 min. Duplicate kinase reactions were preincubated with  $10 \mu\text{M}$  LY294002 for 15 min at room temperature to verify that phosphorylation of all 3 substrates was mediated by PI3K. B, graphic representation of the amount of PIP3 in p85 $\alpha$  +/+ and -/- FLMC before and after KL stimulation for 5 min. Data are expressed as mean  $\pm$  S.E. ( $n = 3$ ). PIP, PtdIns-3-phosphate; PIP2, PtdIns-3,4-bisphosphate; PIP3, PtdIns-3,4,5-trisphosphate.

portion of IgE-dependent signaling. In addition, residual p110 catalytic subunits may be recruited to the membrane by activated Ras (73). However, it seems likely that non-class I<sub>A</sub> PI3Ks are involved, and their identification awaits the availability of the appropriate reagents and strains of deficient mice. In contrast to Fc $\epsilon$ RI-induced exocytosis, our results establish

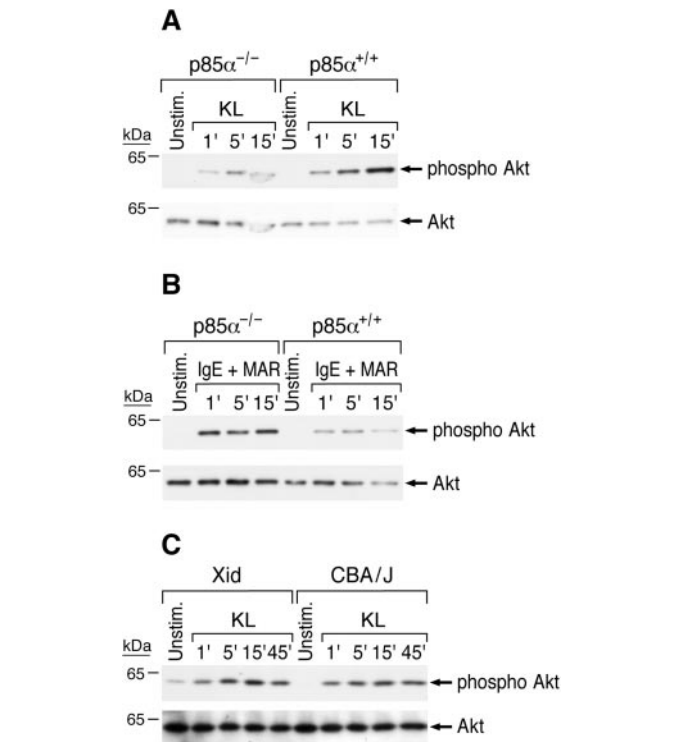


FIG. 7. Effects of p85 $\alpha$  gene deficiency and Xid mutation on KL-dependent Akt activation. Cells were starved as described under “Experimental Procedures” and stimulated as in Fig. 3 with KL (1/100) (A and C) or IgE (5  $\mu\text{g}/\text{ml}$ ) (B) for the indicated times. Whole cell lysates ( $1 \times 10^6$ ) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to Immobilon P membranes. The membranes were immunoblotted sequentially with p-Akt and Akt Abs.

that p85 $\alpha$ -dependent PI3K molecules are essential for maximal exocytosis elicited by KL.

Two key steps leading to exocytosis in mast cells, namely, tyrosine phosphorylation and subsequent calcium mobilization, were not appreciably different in Fc $\epsilon$ RI-activated p85 $\alpha$  +/+ and -/- cells (data not shown), in accordance with the essentially identical levels of Fc $\epsilon$ RI-induced exocytosis (Fig. 3).

However, there were also no notable differences in tyrosine phosphorylation and calcium flux between KL-stimulated p85 $\alpha$  +/+ and -/- FLMC (data not shown), indicating that the deficiency in p85 $\alpha$  -/- cells resulting in inhibition of Kit-induced exocytosis is likely downstream of or parallel to these events. That defect does not appear to involve the mitogen-activated protein kinases, because Kit- (like Fc $\epsilon$ RI) stimulated phosphorylation of c-Jun amino-terminal kinase 1/2, p38, and extracellular signal-regulated kinase 1/2 was not inhibited in the absence of p85 $\alpha$  gene products (data not shown). It is conceivable that the defect in KL-induced Akt phosphorylation observed in p85 $\alpha$  -/- FLMC (Fig. 7A) may relate to the inhibition of exocytosis, particularly in view of the role of Akt in translocation of the glucose transporter GLUT4 to the cell surface in response to insulin receptor signaling (74).

In addition to contributing to Kit-induced exocytosis, a p85 $\alpha$  gene product(s) is required for Kit-induced proliferation of FLMC, because gene disruption inhibited Kit-mediated maintenance of viable FLMC numbers (Fig. 4A) due to a block in cell cycle progression to S phase (Fig. 4C), with attendant inhibition of DNA synthesis (Fig. 4B). Indeed, PI3K has been implicated in promoting cell proliferation and survival (75–78). In particular, mutagenesis and transfection studies indicate that a p85 subunit of PI3K directly binds to tyrosine 719 of Kit, and substitution of this tyrosine with phenylalanine abolishes PI3K activity and impairs cell proliferation and survival in response to KL (47, 48). However, we observed no apparent increase in apoptosis to account for the reduction in Kit-dependent proliferation in p85 $\alpha$  -/- cells (data not shown). This suggests that the role of p85 $\alpha$  gene products in maintaining FLMC proliferation is distinct from their involvement in cell survival signaling. Similarly, Craddock *et al.* (79) dissociated PI3K-directed proliferation from apoptosis in response to IL-3 in BaF/3 cells.

Because Btk is a putative downstream effector of PI3K and because Xid mice show an impairment in B cell development and proliferation similar to that of p85 $\alpha$  -/- mice (59, 66), including defective entry of B cells into the cell cycle (80), we compared the Kit-induced proliferative responses of Xid BMMC to p85 $\alpha$  -/- FLMC. Surprisingly, Kit-dependent proliferation was normal in Xid BMMC, as indicated by the cells' ability to synthesize DNA and progress through the cell cycle, relative to control CBA/J BMMC (Fig. 5). These results suggest that another Tec kinase family member that is expressed in mast cells, such as Itk (81), may substitute for Btk so that a defect in Btk does not inhibit Kit-induced proliferation as much as the absence of p85 $\alpha$  gene products. In contrast, Btk plays a key role in Fc $\epsilon$ RI-dependent responses in mast cells (67), whereas p85 $\alpha$  gene products are dispensable for exocytosis (Fig. 3) and Akt phosphorylation (Fig. 7). Thus, p85 $\alpha$ -dependent PI3Ks and Btk are uncoupled in two receptor systems in mast cells.

Stimulation of p85 $\alpha$  +/+ FLMC by Kit cross-linking resulted in an appreciable increase in phosphotyrosine-associated PI3K activity, which was almost 4-fold lower in p85 $\alpha$  -/- FLMC (Fig. 6). The residual activity was probably associated with p85 $\beta$ . In contrast, there was no appreciable increase in phosphotyrosine-associated PI3K activity with Fc $\epsilon$ RI cross-linking in p85 $\alpha$  +/+ or -/- FLMC (data not shown). Because only class I $_A$  PI3Ks have regulatory subunits with Src homology-2 domains that can bind phosphotyrosines, the data are consistent with the conclusion that essentially all Fc $\epsilon$ RI signaling involves either usage of other PI3K classes or direct activation of p110 catalytic subunits by Ras. Because IL-3-mediated growth of p85 $\alpha$  -/- FLMC was normal but inhibited by LY294002 (Fig. 3 and data not shown), it appears that both Fc $\epsilon$ RI and

IL-3 signaling selectively utilize p85 $\alpha$ -independent PI3K in mast cells.

Stimulation of p85 $\alpha$  +/+ FLMC by either Kit or Fc $\epsilon$ RI cross-linking resulted in a rapid increase in the phosphorylation of Akt (Fig. 7), another downstream effector of PI3Ks (70). The absence of p85 $\alpha$  gene products partially attenuated Kit-induced phosphorylation of Akt, whereas LY294002 substantially inhibited the response, reminiscent of Kit-induced exocytosis and proliferation in p85 $\alpha$  -/- FLMC (Figs. 3 and 4, respectively). These findings indicate that a p85 $\alpha$ -dependent PI3K contributes substantially to maximal induction of all three Kit-mediated responses. In contrast, despite the absence of p85 $\alpha$  gene products and considerably less class I $_A$  PI3K activity in p85 $\alpha$  -/- BMMC, there was no appreciable decrease in the Fc $\epsilon$ RI-induced phosphorylation of Akt (Fig. 7), although the response was largely inhibited with LY294002, suggesting that PI3K molecules without p85 $\alpha$  gene products are selectively involved in Akt phosphorylation after Fc $\epsilon$ RI-induced activation, as is the case for exocytosis (Fig. 3B). In addition, there was no decrease in Kit-elicited Akt phosphorylation in Xid BMMC (Fig. 7), similar to the lack of inhibition of proliferation in these BMMC (Fig. 5).

Thus, our results firmly establish by several criteria a differential usage of PI3K family members in response to two activating agonists that utilize innate (Kit) and adaptive (Fc $\epsilon$ RI) receptors in mast cells and demonstrate a less critical requirement for Btk in the transduction of PI3K signaling in mast cells than in B cells. In addition, the inhibition of Kit-induced exocytosis, proliferation, and Akt phosphorylation in p85 $\alpha$  -/- FLMC may mean that Akt is a newly appreciated downstream effector of PI3K signaling in mast cells in response to KL.

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