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Role of Phosphatidylinositol 3-Kinase in Friend Spleen Focus-Forming Virus-Induced Erythroid Disease▽

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Infection of erythroid cells by Friend spleen focus-forming virus (SFFV) leads to acute erythroid hyperplasia in mice due to expression of its unique envelope glycoprotein, gp55. Erythroid cells expressing SFFV gp55 proliferate in the absence of their normal regulator, erythropoietin (Epo), because of interaction of the viral envelope protein with the erythropoietin receptor and a short form of the receptor tyrosine kinase Stk (sf-Stk), leading to constitutive activation of several signal transduction pathways. Our previous *in vitro* studies showed that phosphatidylinositol 3-kinase (PI3-kinase) is activated in SFFV-infected cells and is important in mediating the biological effects of the virus. To determine the role of PI3-kinase in SFFV-induced disease, mice deficient in the p85α regulatory subunit of class IA PI3-kinase were inoculated with different strains of SFFV. We observed that p85α status determined the extent of erythroid hyperplasia induced by the sf-Stk-dependent viruses SFFV-P (polycythemia-inducing strain of SFFV) and SFFV-A (anemia-inducing strain of SFFV) but not by the sf-Stk-independent SFFV variant BB6. Our data also indicate that p85α status determines the response of mice to stress erythropoiesis, consistent with a previous report showing that SFFV uses a stress erythropoiesis pathway to induce erythroleukemia. We further showed that sf-Stk interacts with p85α and that this interaction depends upon sf-Stk kinase activity and tyrosine 436 in the multifunctional docking site. Pharmacological inhibition of PI3-kinase blocked proliferation of primary erythroleukemia cells from SFFV-infected mice and the erythroleukemia cell lines derived from them. These results indicate that p85α may regulate sf-Stk-dependent erythroid proliferation induced by SFFV as well as stress-induced erythroid hyperplasia.

The Friend spleen focus-forming virus (SFFV) is a highly pathogenic retrovirus that induces rapid erythroblastosis in susceptible strains of mice (for a review, see reference 42). Friend SFFV is a replication-defective virus with deletions in its *env* gene, giving rise to a unique glycoprotein, SFFV gp55. This unique glycoprotein confers pathogenicity to the virus; a vector encoding SFFV gp55 alone is sufficient to induce erythroblastosis in susceptible strains of mice (49). The *Fv-2* gene encodes one of the key susceptibility factors for SFFV-induced erythroid disease (18, 37), as follows: the receptor tyrosine kinase Stk/RON, a member of the Met family of receptor tyrosine kinases (11–12). Susceptibility to SFFV-induced disease is associated with expression of a short form of the receptor tyrosine kinase Stk, termed sf-Stk, that is transcribed from an internal promoter within the *Stk* gene of *Fv-2*-susceptible (*Fv-2*^{ss}) mice but not *Fv-2*-resistant (*Fv-2*^{rr}) mice (37) and is abundantly expressed in erythroid cells (11). Infection of erythroid cells with the polycythemia-inducing strain of SFFV (SFFV-P) induces erythropoietin (Epo)-independent proliferation and differentiation, whereas erythroid cells infected with the anemia-inducing strain of SFFV (SFFV-A) proliferate in the absence of Epo but still require Epo for differentiation (42). Previous studies demonstrated that this Epo-independent erythroblastosis is due to the cell surface interaction of the SFFV envelope protein with the Epo receptor (EpoR) and sf-Stk (31). While interaction with the EpoR appears to be responsible mainly for the induction of Epo-independent differentiation (52), Epo-independent erythroid cell proliferation depends upon activation of sf-Stk. We recently demonstrated that sf-Stk covalently interacts with SFFV-P gp55 in hematopoietic cells that express the EpoR and that this interaction induces sf-Stk activation (31). Furthermore, exogenous expression of sf-Stk, but not a kinase-inactive mutant of sf-Stk, in bone marrow cells from sf-Stk null mice can restore Epo-independent erythroid colony formation in response to SFFV infection (5, 41). Thus, the SFFV envelope glycoprotein induces Epo-independent proliferation of erythroid cells mainly by activating sf-Stk. While sf-Stk is a key susceptibility factor for erythroblastosis induced by both SFFV-P and SFFV-A (18), it is not required for the induction of erythroblastosis by the SFFV mutant BB6, which encodes an envelope glycoprotein, gp42, that is deleted in the membrane-proximal extracellular domain (19) and does not induce sf-Stk activation (31). gp42 of SFFV-BB6 appears to exert its biological effects on erythroid cells by efficiently interacting with the EpoR (9). Compared with wild-type SFFV, SFFV-BB6 causes a relatively indolent and slowly developing disease in mice (19).

A number of signaling pathways normally activated in erythroid cells after erythropoietin (Epo) binds to its cell surface

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receptor (40) are constitutively activated in erythroid cells infected with SFFV. These include JAK/STAT, Ras/Raf/mitogen-activated protein kinase (MAPK), Jun N-terminal kinase, and the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathways (24, 25, 28–30, 32). SFFV gp55 is thought to activate these pathways by interacting with either the EpoR or sf-Stk (17, 31, 43). In several *in vitro* systems, class IA PI3-kinase has been shown to be activated by Epo through the EpoR (8, 20, 21) or by SFFV through sf-Stk (5, 14). We and others have shown that the PI3-kinase pathway is important for the induction of Epo independence by SFFV (5, 29). The class IA subclass of PI3-kinase is a heterodimer comprising the p110 (α, β, δ) catalytic unit and one of five regulatory subunits (85α, p55α, p50α, 85β, and 55γ) (15). The first 3 regulatory subunits are all splice variants of the same gene (*pik3r1*). Deletion of *pik3r1*, which encodes p85α, p55α, and p50α, is lethal (6, 7), and these regulatory subunits of PI3-kinase are required for normal murine fetal erythropoiesis in mice (10).

To determine the role of p85α in SFFV-induced erythroleukemia, we used a distinct nonlethal *pik3r1* knockout mouse which lacks only the p85α regulatory subunit of PI3-kinase (45, 47), allowing the study of SFFV-induced erythroleukemia in adult mice. Our results indicate that p85α regulates SFFV-induced erythroid hyperplasia induced *in vivo* by sf-Stk-dependent, but not sf-Stk-independent, isolates of the virus as well as stress-induced erythropoiesis and suggest that this regulation may occur through the interaction of sf-Stk with p85α.

MATERIALS AND METHODS

Mouse and virus inoculation. $p85\alpha^{+/-}$ mice were obtained from RIKEN BRC. $p85\alpha^{-/-}$ mice (45, 47) were backcrossed to BALB/c mice for 12 generations before intercrossing them with heterozygous mice. The colony has been maintained in our animal facility under specific pathogen-free conditions by mating $p85\alpha^{-/-}$ male mice and $p85\alpha^{+/-}$ female mice. Two of the three isoforms of *pik3r1*, p55α and p50α, are intact in these mice (45, 47). $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mice were inoculated intravenously with the following three different strains of SFFV: *Fv-2*-restricted Friend SFFV-P, obtained from $SFFV_{AP}$-L-transfected NIH 3T3 cells superinfected with Friend murine leukemia virus (F-MuLV) (49); *Fv-2*-restricted Friend SFFV-A, obtained from $SFFV_A$-L-transfected NIH 3T3 cells that had been superinfected with F-MuLV (49); and *Fv-2*-independent SFFV-BB6, obtained from infecting SFFV-BB6 clone 13 NIH 3T3 cells with F-MuLV (27). After 14 days, mice were sacrificed for further analysis. All experiments were performed in accordance with our universities' guidelines.

Phenylhydrazine (PHZ) treatment. Mice were treated intraperitoneally with phenylhydrazine (60 mg/kg at 0 and 24 h), and the mice were sacrificed and investigated on day 5.

Primary erythroleukemic cells and cell lines. Primary erythroleukemic cells were prepared by injection of F-MuLV/Friend SFFV-P into NIH Swiss mice, as previously described (41). 293T, NIH 3T3/SFFV-P, NIH 3T3/SFFV-A, and NIH 3T3/SFFV-BB6 cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (FCS). Mouse erythroleukemia cell lines NP1, NP4, and NP7 were established from mice infected with helper-free Friend SFFV-P (50). These cell lines were maintained in Dulbecco's minimal essential medium supplemented with 10% FCS.

Protein analysis. Cell lysates were prepared by resuspending cells in lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM Na_3VO_4, 1 μg/ml each of aprotinin and leupeptin), followed by incubation on ice for 20 min. Insoluble components were removed by centrifugation, and protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories). Proteins were separated by electrophoresis on Tris-glycine minigels and then transferred electrophoretically to nitrocellulose filters for Western blotting with rat anti-SFFV *env* monoclonal antibody (7C10) (48); rabbit anti-p85α, anti-phospho-Akt, and anti-Akt (Cell Signaling Technology, Beverly, MA); or goat anti-actin (Santa Cruz Biotechnology). Blots were incubated with anti-rat IgG antibody conjugated to horseradish peroxidase (HRP) (Cell Signaling Technology, Beverly, MA), anti-rabbit IgG antibody conjugated to HRP (GE Healthcare), or anti-goat IgG antibody conjugated to HRP (Santa Cruz Biotechnology), followed by visualization using enhanced chemiluminescence (ECL) (GE Healthcare). For immunoprecipitation assays, cell lysates were immunoprecipitated with mouse anti-hemagglutinin (HA) antibody (Roche Diagnostics, Indianapolis, IN) at 4°C overnight. Immune complexes were then collected with protein G-agarose (Santa Cruz Biotechnology) and transferred electrophoretically to nitrocellulose filters. The filters were then blotted with mouse anti-myc conjugated with HRP (Wako Pure Chemical Industries, Osaka, Japan) or mouse anti-HA followed by anti-mouse IgG conjugated to HRP (GE Healthcare) before using the ECL Western blotting system to detect bound proteins. Quantification of phospho-Akt was determined by scanning filters using ImageJ 1.37v software and determining the ratio of phospho-Akt to total Akt.

Plasmids. pEFsf-Stk (wild-type) and pEFsf-Stk K190M (kinase-inactive mutant) expression vectors tagged with N-terminal myc were described previously (27). pEFsf-StkY429F and pEFsf-StkY436F mutant vectors, with each tyrosine (Y)-to-phenylalanine (F) substitution within sf-Stk, were generated by PCR amplification with the following primers (mutated codons are underlined): for pEF sf-StkY429F construction, Y429F-S (5′-TCGGATCCAACATGACCGTG GGTGGTGAGGTCTGCC-3′) and Y429F-R (5′-TAAGCTGCTGTCAGCTG CACAAAGTGGTCCCCAAGCAGTG-3′); and for pEFsf-StkY436F construction, Y436F-S (5′-GTGCAGCTGACAGCAGCTTTTGTGAACGTAGGCCC CAG-3′) and Y436F-R (5′-AGAATTCCAAGTGGGCAGGGGTGGCTCTGA GAGAGGC-3′). Each PCR-mutated fragment was replaced with each normal fragment of pEFsf-Stk. HA-tagged p85α was kindly provided by T. Kadowaki (University of Tokyo).

Transfection. 293T cells plated in 60-mm dishes were transfected with $SFFV_{AP}$-L (49) and p85α plasmids along with either the pEFsf-Stk, pEFsf-StkK190M, pEFsf-StkY429F, or pEFsf-StkY436F plasmids using Lipofectamine 2000 reagent (Invitrogen). Approximately 48 h after transfection, cells were collected and further analyzed.

Inhibitors. The protein kinase inhibitor LY294002 was purchased from Calbiochem (Darmstadt, Germany). The inhibitor was reconstituted in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO).

Cell proliferation assay. Cell proliferation was assayed using the WST-1 reagent (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's instructions and as previously described (30). Cells were plated in 96-well microtiter plates with various concentrations of the inhibitor in vehicle or with vehicle alone and incubated for 24 h before the addition of the cell proliferation reagent WST-1. After incubation of the samples in a humidified atmosphere, the absorbance of samples was measured at 450 nm against that of background controls using an enzyme-linked immunosorbent assay reader.

Flow cytometric analysis. Phycoerythrin (PE)-conjugated anti-Ter119 antibody (eBioscience), fluorescein isothiocyanate (FITC)-conjugated anti-Ter119 antibody (Miltenyi Biotec), and their PE rat IgG2b isotype controls (eBioscience) and FITC rat IgG2b isotype controls (Medical & Biological Laboratories) were used for cell surface phenotyping. Spleen mononuclear cells prepared using Histopaque (Sigma-Aldrich) were incubated with antibody for 30 min at 4°C before being washed in phosphate-buffer saline (PBS) containing 1% fetal bovine serum (FBS) and fixed with 1% formaldehyde in PBS. The cells were analyzed using the FACScan flow cytometer.

Statistical analysis. Statistical analysis was performed by using the nonparametric Mann-Whitney U test.

RESULTS

Role of p85α in steady-state and stress erythropoieses in adult mice. It is known that the p85α regulatory subunit of class IA PI3-kinase is required for normal murine fetal erythropoiesis (10), but it is not clear if it is required for normal adult erythropoiesis in the mouse. The availability of a nonlethal knockout mouse model for *pik3r1* in which only the p85α regulatory subunit of PI3-kinase is deleted allowed us to investigate the role of p85α in steady-state and stress erythropoieses in adult mice. When untreated, age-matched $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mice were examined for spleen weights and the percentages of Ter119-positive erythroid cells in the spleens (Fig. 1A), as well as for hematocrit (Ht) levels (data not shown). No significant differences could be detected, indicating that steady-state erythropoiesis was not affected by p85α sta-

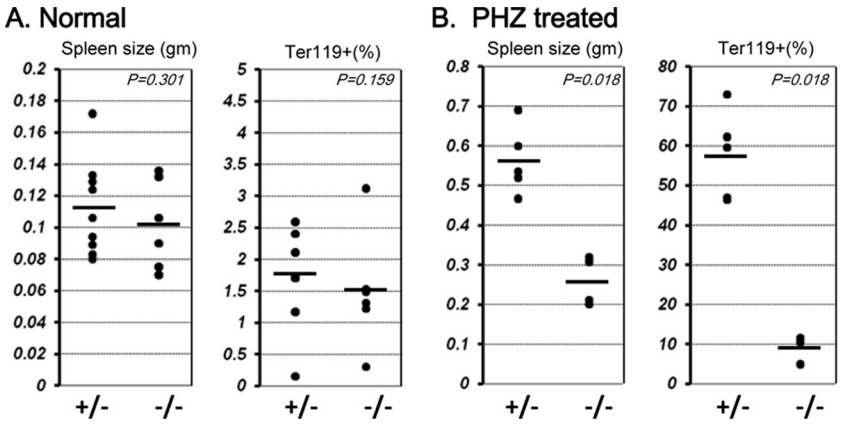

FIG. 1. Erythropoiesis in untreated and phenylhydrazine-treated p85α-deficient mice. Age-matched p85α$^{+/-}$ mice or p85α$^{-/-}$ mice were treated with phenylhydrazine (PHZ), and after 5 days, the increase in splenic erythropoiesis due to hemolytic anemia was investigated by determining the spleen weights (g) and frequencies of Ter119-positive cells (%). (A) Untreated p85α$^{+/-}$ mice (n = 9) or p85α$^{-/-}$ mice (n = 6); (B) PHZ-treated p85α$^{+/-}$ mice (n = 5) or p85α$^{-/-}$ mice (n = 4). Statistical analysis was performed by using the nonparametric Mann-Whitney U test.

tus. In contrast, p85α$^{+/-}$ and p85α$^{-/-}$ mice treated with phenylhydrazine (PHZ), which causes hemolytic anemia, showed a significant difference in their response to erythropoietic stress (Fig. 1B) While p85α$^{+/-}$ mice treated with PHZ showed a large increase in spleen weights (range, 0.47 to 0.69 g; average, 0.56 g) and frequencies of Ter119^{+} cells (range, 46.4 to 73%; average, 57.7%) in the spleen to compensate for the hemolytic anemia induced, the spleens of p85α$^{-/-}$ mice treated with PHZ failed to support efficient erythroid hyperplasia, as indicated by significantly lower spleen weights (range, 0.2 to 0.32 g; average, 0.26 g) and frequencies of Ter119^{+} cells (range, 5.0 to 11.6%; average, 9.6%). Thus, p85α-deficient mice are unable to support stress-induced erythropoiesis, consistent with previous data indicating that a functional p85α gene is required for normal murine fetal erythropoiesis (10) and that BMP4/Smad5-dependent stress erythropoiesis is required for the expansion of erythroid progenitors during fetal development (39).

Role of p85α in SFFV-induced erythroleukemia. Many signal transduction pathways are constitutively activated in hematopoietic cells infected with Friend SFFV, including the PI3-kinase pathway. Since Friend SFFV has been shown to utilize the BMP4-dependent stress erythropoiesis pathway to induce erythroleukemia (44), and our data shown above indicate that p85α is required for the response of mice to erythropoietic stress, we carried out studies to determine if the p85α regulatory subunit of PI3-kinase plays a role in SFFV-induced erythroid hyperplasia. For these studies, adult p85α$^{+/-}$ and p85α$^{-/-}$ mice (both strains carry the *Fv-2*^{ss} genotype that expresses sf-Stk) were inoculated intravenously with the following three different strains of SFFV: sf-Stk-dependent Friend SFFV-P and SFFV-A and the sf-Stk-independent mutant SFFV-BB6. After 14 days, mice were analyzed for erythroid hyperplasia, as indicated by spleen weights and the percentages of Ter119-positive erythroid cells in the spleens. As an indicator of erythroid cell differentiation, hematocrit (Ht) values were also determined. Uninfected adult p85α$^{+/-}$ mice and p85α$^{-/-}$ mice exhibited no difference in spleen weights and the percentages of erythroid cells in the spleens (Fig. 1A).

p85α$^{+/-}$ mice infected with SFFV-P showed both splenomegaly (spleen size, 1.6 to 2.8 g; average, 2.04 g) and polycythemia (Ht, 37 to 55%) (Fig. 2A). Although the spleens from SFFV-P-infected p85α$^{-/-}$ mice also showed an increase in size (range, 1.1 to 1.6 g; average, 1.4 g), this increase was significantly (P = 0.003) smaller than that in virus-infected p85α$^{+/-}$ mice. Like SFFV-P-infected p85α$^{+/-}$ mice, p85α$^{-/-}$ mice infected with SFFV-P also showed polycythemia (Ht, 44 to 61.5%), and interestingly, these values were even higher than those in SFFV-P-infected p85α$^{+/-}$ mice (Fig. 2A). When the spleen cells were analyzed for the presence of the erythroid cell surface marker Ter119, those from SFFV-P-infected p85α$^{+/-}$ mice expressed higher percentages of Ter119-positive cells (range, 63.9 to 88.7%; average, 78.8%) than virus-infected p85α$^{-/-}$ mice (range, 40.1 to 70.4%; average, 60.7%) (Fig. 2A). The reduction in the frequencies of Ter119-positive cells correlates with the significantly reduced spleen sizes in the p85α$^{-/-}$ mice, suggesting that p85α may regulate the SFFV-P-induced proliferation of erythroleukemic cells.

Similar results were obtained from mice infected with SFFV-A (Fig. 2B). Spleens from SFFV-A-infected p85α$^{+/-}$ mice showed a significant increase in size (range, 1.1 to 2.5 g; average, 1.9 g) compared with those from uninfected p85α$^{+/-}$ mice. Although p85α$^{-/-}$ mice also showed an increase in spleen size (range, 0.9 to 1.5 g; average, 1.2 g) after infection with SFFV-A, the increase was significantly (P = 0.011) less than that in SFFV-A-infected p85α$^{+/-}$ mice. The frequencies of Ter119-positive cells in the spleens of SFFV-A-infected p85α$^{+/-}$ mice ranged from 53.0 to 75.6% but in p85α$^{-/-}$ mice, ranged only from 31.5 to 53.4% (Fig. 2B). Thus, the reduction in Ter119-positive cells correlates with the decrease in spleen size in the SFFV-A-infected p85α$^{-/-}$ mice. All of the mice infected with SFFV-A were anemic (Fig. 1B), with p85α$^{+/-}$ mice showing Ht values of 20 to 39% and p85α$^{-/-}$ mice showing values of 27 to 41%. Thus, p85α expression appears to play a role in regulating the proliferation, but not the differentiation, of erythroleukemia cells induced by both SFFV-P and SFFV-A.

The induction of erythroleukemia by both SFFV-P and

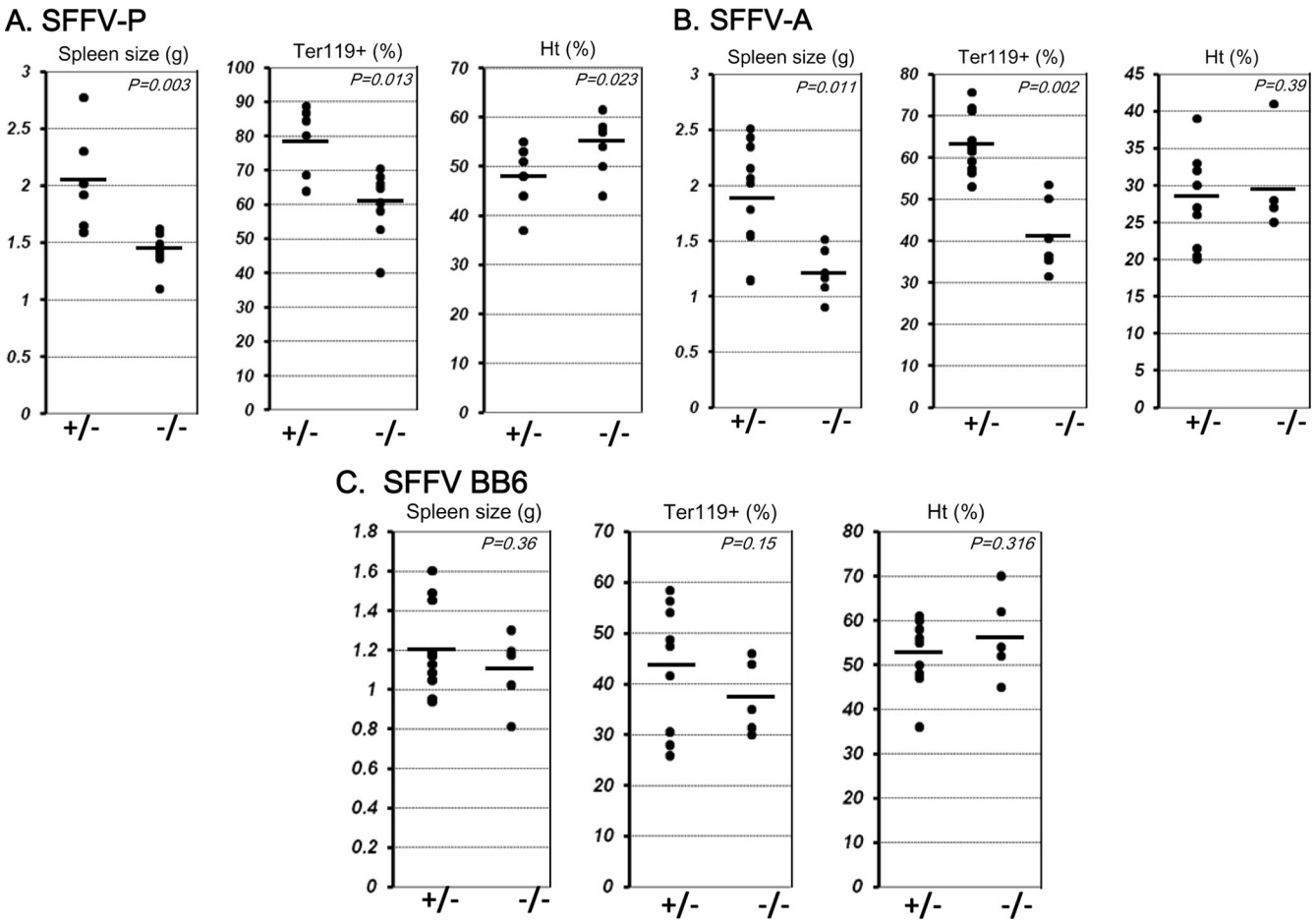

FIG. 2. SFFV-induced erythroleukemia in p85α-deficient mice. Adult $p85\alpha^{+/-}$ or $p85\alpha^{-/-}$ mice were injected with 3 different strains of SFFV and analyzed 14 days later for spleen weights in grams (g), frequencies of Ter119-positive cells (%), and hematocrit (Ht) levels (%). (A) $p85\alpha^{+/-}$ mice ($n = 6$) or $p85\alpha^{-/-}$ mice ($n = 9$) were inoculated intravenously at 5 to 8 weeks of age with 0.3 ml of SFFV-P-containing virus preparations; (B) $p85\alpha^{+/-}$ mice ($n = 11$) or $p85\alpha^{-/-}$ mice ($n = 6$) (all 7 to 11 weeks old) were inoculated intravenously with 0.3 ml of SFFV-A-containing virus preparations; (C) $p85\alpha^{+/-}$ mice ($n = 10$) or $p85\alpha^{-/-}$ mice ($n = 5$) (all 7 weeks old) were inoculated intravenously with 0.3 ml SFFV-BB6-containing virus preparations. Statistical analysis was performed by using the nonparametric Mann-Whitney U test. Horizontal bars indicate mean values.

SFFV-A is dependent upon the *Fv-2* genotype of the mouse and occurs only in *Fv-2*^{ss} mice, which express the receptor tyrosine kinase sf-Stk. To determine if p85α also plays a role in the erythroleukemia induced by an sf-Stk-independent mutant of SFFV, we infected $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mice with SFFV-BB6. As shown in Fig. 2C, both groups of mice developed splenomegaly, with similar spleen weights (0.9 to 1.6 g for $p85\alpha^{+/-}$ mice and 0.8 to 1.3 g for $p85\alpha^{-/-}$ mice). The frequencies of Ter119-positive cells between the SFFV-BB6-infected $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ groups (25.8 to 58.5% and 30 to 46%, respectively) were not significantly different. Both groups showed polycythemia, with no significant differences in Ht levels in the $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mice (36 to 61% and 45 to 70%, respectively) (Fig. 3C), indicating that p85α status did not affect erythroid cell differentiation. Thus, unlike erythroleukemia induced by sf-Stk-dependent SFFV-P and SFFV-A, erythroleukemia induced by sf-Stk-independent SFFV-BB6 does not appear to be regulated by p85α.

Examination of splenic lysates using Western blot analysis for p85α confirmed that p85α was expressed in the virus-infected $p85\alpha^{+/-}$ mice but not in the $p85\alpha^{-/-}$ mice (Fig. 3A). Furthermore, the levels of expression of phosphorylated Akt, a downstream component of the PI3-kinase signaling pathway, were reduced in the spleens of the virus-infected $p85\alpha^{-/-}$ mice compared with those levels in the spleens of $p85\alpha^{+/-}$ mice (Fig. 3B). Importantly, spleens from the virus-infected mice showed high levels of expression of their respective SFFV envelope proteins, with no significant differences in the levels of expression in $p85\alpha^{+/-}$ and $p85\alpha^{-/-}$ mice (Fig. 3C).

Interaction of sf-Stk with p85α. Since our data indicate that p85α status is more important for the induction of erythroleukemia by SFFV isolates that are sf-Stk-dependent than by those that are sf-Stk independent, we carried out studies to determine whether sf-Stk was interacting with p85α. 293T cells transfected with plasmids expressing myc-tagged sf-Stk, $SFFV_{AP}$-L, and HA-tagged p85α were immunoprecipitated with anti-HA antibody to precipitate p85α and then immunoblotted with anti-myc antibody conjugated with HRP for detection of sf-Stk. As shown in Fig. 4, when anti-HA p85α

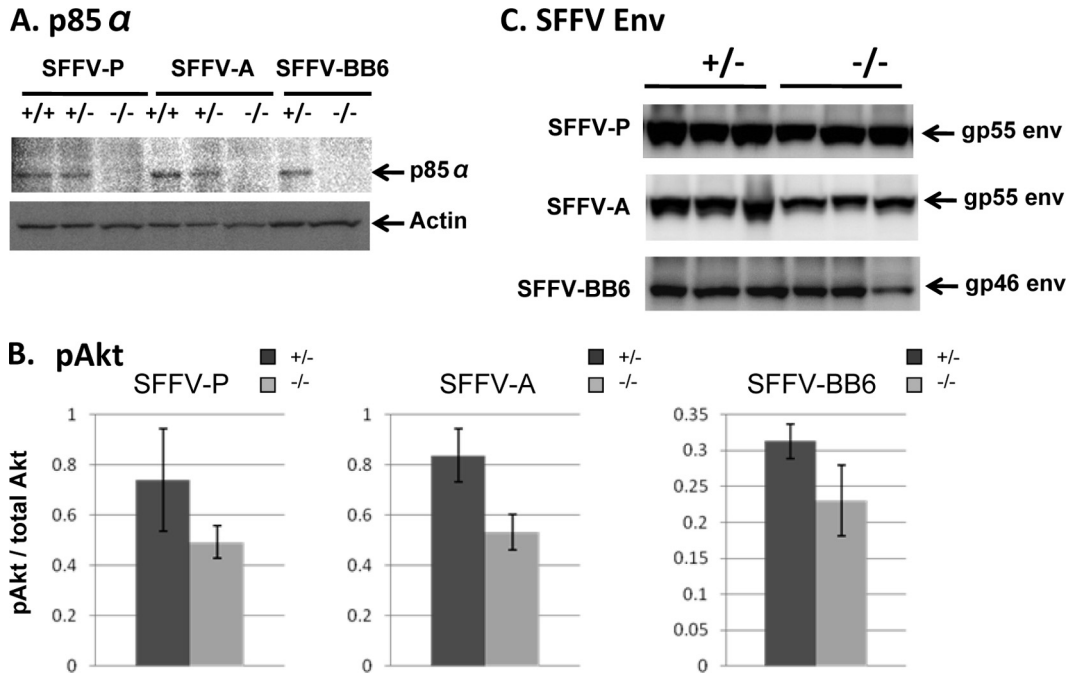

FIG. 3. Expression of viral and cellular proteins in SFFV-infected p85α-deficient mice. Spleen cell lysates were prepared from $p85\alpha^{+/-}$ or $p85\alpha^{-/-}$ mice infected with SFFV-P, SFFV-A, or SFFV-BB6 and examined by Western blot analysis for expression of p85α (A), expression of phosphorylated Akt (pAkt), as represented by the mean expression level of phosphorylated Akt/expression level of total Akt ratio in individual spleens ± SD (B), and expression of SFFV Env (C).

immunoprecipitates were immunoblotted with anti-myc, a band corresponding to the sf-Stk wild type was detected (lane 1), indicating that sf-Stk and p85α are interacting. p85α failed to interact strongly with a kinase-inactive mutant of sf-Stk (K190M) (lane 4) or with an sf-Stk construct in which tyrosine 436 in the multifunctional docking site of sf-Stk had been mutated to phenylalanine (lane 3), a change which was previously shown to be important for the induction of Epo independence by sf-Stk in conjunction with SFFV (5, 41). Mutation of tyrosine 429 in sf-Stk, which was previously shown to have little effect on the induction of Epo independence by sf-Stk in conjunction with SFFV (5, 41), did not affect its ability to interact with p85α (lane 2). These results indicate that sf-Stk interacts with p85α and that kinase activity and tyrosine 436 of sf-Stk contribute to the interaction.

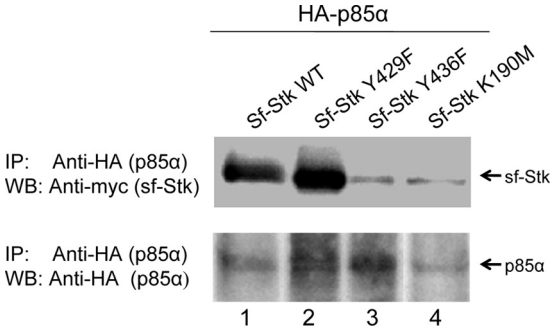

FIG. 4. Interaction sf-Stk with p85α. 293T cells were transfected with $SFFV_{AP}$-L, HA-tagged p85α, and the indicated myc-tagged sf-Stk-expressing plasmids. Cell lysates were immunoprecipitated (IP) with anti-HA to precipitate p85α, followed by immunoblotting (Western blotting [WB]) with HRP-conjugated anti-myc antibody to detect sf-Stk or with anti-HA- and anti-HRP-conjugated anti-rabbit IgG to detect p85.

Proliferation of both primary and immortal erythroleukemia cells from Friend SFFV-P-infected mice is inhibited by treatment with a PI3-kinase inhibitor. Friend SFFV-induced erythroleukemia occurs in two stages. In the first stage, Friend SFFV causes Epo-independent erythroid proliferation and differentiation. Primary erythroleukemic splenocytes from these animals can grow in liquid culture for only a short time but can both proliferate and differentiate in semisolid medium, forming hemoglobin-positive erythroid colonies. In the second stage, Friend SFFV-infected erythroid cells become blocked in differentiation, resulting in the outgrowth of transformed erythroleukemia cells that can be grown as cell lines. While we could establish erythroleukemia cell lines from SFFV-P-infected p85α heterozygous mice, we failed to do so with SFFV-P-infected p85α-deficient mice (data not shown), suggesting that PI3-kinase activity is important for establishing and maintaining immortal erythroleukemia cell lines from SFFV-infected mice. In support of this, we showed that the PI3-kinase inhibitor LY294002 not only inhibited the growth of primary erythroleukemic splenocytes from a Friend SFFV-P-infected mouse but also inhibited the growth of immortal erythroleukemia cell lines derived from these mice (Fig. 5). Growth inhibition occurred as early as 24 h after exposure in a dose-dependent fashion, with kinetics similar to that seen using Friend SFFV-P-infected HCD-57 cells, as previously reported (29). These results indicate that blocking PI3-kinase activity in primary and immortal erythroleukemia cells results in inhibition of cell growth and suggests that PI3-kinase plays an im-

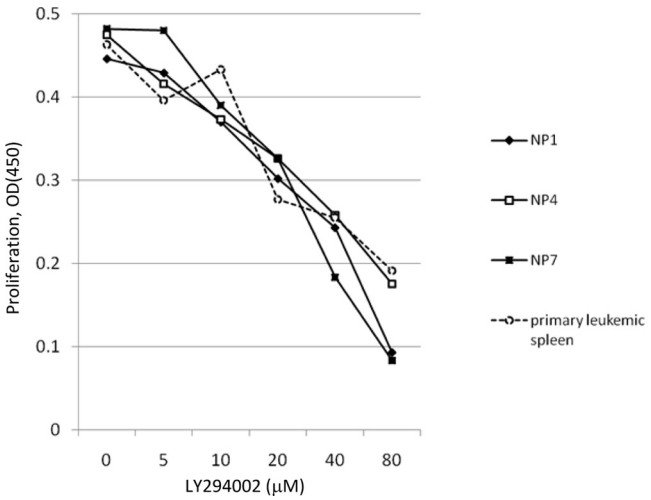

FIG. 5. Effects of a PI3-kinase inhibitor on the proliferation of primary erythroleukemia cells and erythroleukemia cell lines from Friend SFFV-infected mice. Erythroleukemia cells were cultured with different concentrations of the PI3-kinase inhibitor LY294002 for 24 h. Proliferation was then measured using the WST-1 reagent. The cells analyzed were primary leukemic spleen cells (○) and the erythroleukemia cell lines NP1 (◆), NP4 (□), and NP7 (■). The graph represents the mean results obtained from using triplicate samples. The standard error was less than 0.05. OD_{450}, optical density at 450 nm.

portant role in leukemic cell survival. Interestingly, PI3-kinase also appears to play an important role in the survival of leukemic cells from SFFV-BB6-infected mice, whose proliferation was inhibited *in vitro* by the PI3-kinase inhibitor LY294002 (data not shown). Since p85α status had no effect on erythroid hyperplasia induced in mice by SFFV-BB6, the PI3-kinase inhibitor results suggest that other subunits of p85 may be contributing to PI3-kinase activity in SFFV-BB6-infected $p85\alpha^{-/-}$ mice.

DISCUSSION

This study analyzed the role of class IA PI3-kinase in erythroleukemia induced by sf-Stk-dependent and -independent strains of SFFV in mice which lack the p85α regulatory subunit of this enzyme. Our data indicated that mice lacking p85α showed a significant reduction in the extent of erythroid hyperplasia induced by the sf-Stk-dependent strains SFFV-P and SFFV-A but not induced by the sf-Stk-independent strain SFFV-BB6.

Class IA PI3-kinase has been shown to be activated by Epo through the EpoR (8, 20, 21), either by direct binding or through insulin receptor substrate (IRS)-related adapter molecules (2, 29). Hematopoietic cells infected with SFFV show constitutive activation of PI3-kinase, and SFFV is thought to activate PI3-kinase through sf-Stk or the EpoR primarily via IRS-related adaptor molecules (14, 29). In this study, we showed that sf-Stk interacts with p85α, but the interaction requires sf-Stk kinase activity since a kinase-inactive mutant of sf-Stk fails to interact with p85α. We further found that tyrosine 436 in the multifunctional docking site of sf-Stk was also required for interaction with p85α. Previous studies have shown that after growth factor stimulation, the Src homology 2 (SH2) domain of p85α binds to YXXM motifs of phosphorylated tyrosines in activated receptors or adaptor molecules (1, 4, 33). Since tyrosine 436 of sf-Stk is not part of a YXXM motif, it is more likely that p85α indirectly interacts with sf-Stk via adaptor proteins which bind to tyrosine 436 on the kinase. It has been shown that the adapter protein Grb2 binds to tyrosine 436 on sf-Stk and that Grb2 recruits the IRS adapter protein Gab2 to sf-Stk (46). Gab2 contains multiple p85α binding sites (26), and our previous study showed that SFFV activates PI3-kinase via Gab2 (29). Previous studies have shown that tyrosine 436 in sf-Stk is required for the development of Epo-independent erythroid colonies (5, 41).

Erythroid hyperplasia in SFFV-P- and SFFV-A-infected wild-type mice appears to be determined by both EpoR-mediated and sf-Stk-mediated signaling. This is in contrast to the BB6 mutant of SFFV, which causes polycythemia and splenomegaly in the absence of sf-Stk signaling. Previous studies showed that SFFV-BB6 does not interact with sf-Stk (31), most likely due to a large deletion in its envelope protein that includes cysteines that may be necessary for interaction with sf-Stk. SFFV-BB6, however, appears to interact strongly with the EpoR, causing its superactivation, and it is this interaction that is likely responsible for its ability to induce Epo-independent erythroid hyperplasia (9, 16). SFFV-P and SFFV-A are more pathogenic to mice than SFFV-BB6, causing extensive erythroid hyperplasia that rapidly develops into erythroleukemia. In contrast, SFFV-BB6 causes a more indolent disease associated with less robust erythroid hyperplasia that rarely develops into erythroleukemia (19). Activation of the PI3-kinase pathway by SFFV-P and SFFV-A can occur through both the EpoR and sf-Stk, and the signals generated most likely combine to control erythroid proliferation and thus splenomegaly *in vivo*. We propose that erythroid hyperplasia induced by sf-Stk signaling is dependent upon p85α, while EpoR signaling is not. Thus, sf-Stk-dependent viruses such as SFFV-P and SFFV-A induce less splenomegaly in $p85\alpha^{-/-}$ mice, because the lack of p85α blocks sf-Stk signaling. In contrast, spleen size is not determined by p85α status in SFFV-BB6-infected mice, because this sf-Stk-independent virus depends solely on EpoR-mediated signaling, which does not appear to be dependent upon p85α.

Our data also indicate that stress erythropoiesis is regulated by p85α. This is consistent with data showing that BMP4-dependent stress erythropoiesis is required for expansion of erythroid progenitors during fetal development (39) and that p85α is required for normal fetal erythropoiesis (10). It was recently reported that Friend SFFV utilizes the BMP4-dependent stress erythropoiesis pathway to induce erythroleukemia (44) Thus, the significantly reduced erythroid hyperplasia observed in SFFV-infected mice deficient in p85α may be due to the failure of these mice to activate this stress pathway. Since our data indicate that erythroid hyperplasia induced in mice by SFFV-BB6 does not depend upon p85α status, perhaps SFFV-BB6-induced erythroid hyperplasia does not utilize the BMP4-dependent stress erythropoiesis pathway.

Mutations of the p85α regulatory subunit of PI3-kinase have been reported in human cancers (3, 13, 34, 38, 51) such as breast, colon, ovarian, glioblastoma, and pancreatic, and these mutations promote tumorigenesis through class IA PI3-kinase activation (13). In this study we showed that activation of the

PI3-kinase pathway contributes to the development of erythroid hyperplasia and erythroleukemia. We failed to establish erythroleukemia cell lines from SFFV-infected p85α-deficient mice but not p85α heterozygous mice. Erythroleukemia cell lines derived from mice infected with Friend SFFV inappropriately express the myeloid transcription factor PU.1 due to proviral insertion (22, 23, 35, 36). Therefore, activation of the PI3-kinase pathway through sf-Stk may regulate PU.1 activation by SFFV. Blocking PI3-kinase activity with a pharmacological inhibitor inhibited the growth of both primary erythroleukemia cells induced by SFFV-P and the immortal erythroleukemia cell lines derived from them. Taken together, our results indicate that PI3-kinase through its p85α regulatory subunit plays an important role in stress erythropoiesis and in establishing and maintaining sf-Stk-dependent erythroid hyperplasia and erythroleukemia induced in mice by Friend SFFV.

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