Sasaki et al. www.jem.org/cgi/doi/10.1084/jem.20041891

SUPPLEMENTAL MATERIALS AND METHODS

Generation of PIPKIα-deficient mice. E14K murine embryonic stem (ES) cells heterozygous for a deletion mutation of the *PIPKIα* gene were generated by replacing 1.7 kb of the murine *PIPKIα* gene (including the region encoding the NH_2-terminal amino acids 68–106 indispensable for PIPKI kinase activity) with a *PGK-Neo* cassette. The targeting vector contained a 12-kb genomic murine (129/Ola) *PIPKIα* fragment plus the *PGK-Neo* cassette inserted in antisense orientation to *PIPKIα* transcription. The linearized construct was electroporated into 10^7 E14K ES cells. ES cell colonies resistant to G418 (0.3 mg/ml^{-1}) were screened for homologous recombination by PCR. Recombinant colonies were confirmed by hybridizing a Southern blot of HindIII- and EcoRV-digested genomic DNA to a 440 bp 3′ flanking probe. Eight different targeted ES cells derived from seven independent electroporation experiments were injected into C57BL/6 blastocysts. Chimeric male mice were crossed with C57BL/6 females to achieve germline transmission. After heterozygous matings, *PIPKIα*$^{-/-}$ mice were distinguished from *PIPKIα*$^{+/-}$ and *PIPKIα*$^{+/+}$ mice by Southern blotting. PIPKI isoforms were detected by Western blotting using antibody (Ab) specific for PIPKIα (Santa Cruz Biotechnology, Inc.), PIPKIβ (Upstate Biotechnology) or PIPKIγ (raised against a peptide SDEEDAPSTDIYF from the COOH-terminal part of PIPKIγ). Littermates were used as controls for all experiments. Equivalent results and phenotypes were obtained for *PIPKIα*$^{-/-}$ mice derived from two independent targeted ES cell clones.

Surface expression of FcεRI. Surface expression of c-Kit was analyzed using PE-conjugated anti–c-Kit Ab (BD Biosciences). For FcεRI expression, cells were incubated with anti–p-DNP IgE mAb (clone SPE-7; Sigma-Aldrich), followed by FITC-conjugated anti–mouse IgE (BD Biosciences). Samples were analyzed by flow cytometry using a FACScan (Becton Dickinson).

RT-PCR. 10^7 BMMCs sensitized with anti-DNP IgE were stimulated with DNP-HSA (50 ng/ml^{-1}). Total RNA was prepared using TRIzol reagent (GIBCO BRL), and first-strand cDNA was synthesized using 5 μg of total RNA with M-MLV Reverse Transcriptase (TOYOBO). Specific PCR primers used were as follows: IL-2, forward, 5′-TGGAGCAGCTGTTGATGGACCTAC-3′, reverse, 5′-AGATGATGCTTTGACAGAAGGCTATC-3′; IL-3, forward, 5′-TGGAGGACCAGAACGAGACAATG-3′, reverse, 5′-TTCGCAGATGTAGGCAGGCAAC-3′; IL-4, forward, 5′-ATCGGCATTTTGAAC-GAGGTC-3′, reverse, 5′-CAGTGATGTGGACTTGGACTCATTC-3′; TNF-α, forward, 5′-GAAAAGCAAGCAGCCAACCAG-3′, reverse, 5′-TTCATCTGTCCAACCCACGG-3′; and GAPDH, forward, 5′-CAACGACCCCTTCATTGACCTC-3′, reverse, 5′- ATCCACGACGGACACAT-TGG-3′. Amplification conditions for cytokine gene expression were as follows: 94°C for 0.5 min, 98°C for 0.5 min; 28 cycles (GAPDH, TNFα) or 30 cycles (IL-2, -3, -4) of 94°C for 0.5 min, 65°C for 0.5 min, 72°C for 0.75 min; 72°C for 7 min; and 4°C for 2 h (PTC-100 thermal controller; MJ Research). The amplification of cytokines showed a linear increase in detectable levels with corresponding increases in cDNA, allowing a relative quantitation of cytokine mRNA levels normalized to the expression of the housekeeping gene GADPH.

Actin cytoskeleton assessments. Flow cytometry was used to quantitate the relative amount of F-actin per BMMC. BMMCs preloaded with anti-DNP IgE were stimulated with DNP-HSA (50 ng/ml^{-1}) and fixed by adding 2 vol. 7.4% formaldehyde in PBS. Fixed cells were washed and incubated with PBS containing 0.2 U/ml^{-1} Alexa Fluor 488 phalloidin (Molecular Probes), 0.1% lysophosphatidylcholine (Nacalai Tesque), and 1% BSA for 1 h at room temperature. Triplicate samples were analyzed using a FACScan (Becton Dickinson). A minimum of 5×10^3 BMMC events was recorded and used to calculate the mean phalloidin fluorescence intensity (1). For microscopic analysis, stimulated cells were washed and fixed with 2.4% paraformaldehyde in PBS for 1 h at room temperature. The fixed cells were incubated with PBS containing 0.2 U/ml^{-1} Alexa Fluor 488 phalloidin, 0.1% lysophosphatidylcholine, and 1% BSA for 1 h at room temperature. Fluorescence images were acquired on a DM IRE2 Leica microscope fitted with a confocal imaging system (Yokogawa). This system comprises a dual wavelength argon/krypton laser and a CSU-21 confocal scanning unit. Images were acquired with no pixel binning by an Orca-ER cooled charge-coupled device camera (Hamamatsu) driven by IPLab software version 6.0 (Scanalytics) and processed on Macintosh G4 computers using IPLab and Photoshop 7.0.1 software (Adobe Systems). Excitation was at 488 nm and emission was collected with a band-pass filter transmitting between 500 and 525.

Immunoblotting and immunoprecipitation. BMMCs were sensitized with anti-DNP IgE (0.2 μg/ml^{-1}; SPE-7) for 15 h. After washing and resuspension in OPTI-MEM containing 0.1% BSA, the cells ($5–10 \times 10^6$) were stimulated with DNP-HSA (50 ng/ml^{-1}). Reactions were terminated by the addition of ice-cold PBS and cell lysates were prepared in RIPA buffer (1% Triton X-100, 10 mM Tris/HCl, pH 7.4, 150 mM NaCl, 30 mM $Na_4P_2O_7$, 5 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, and a protease inhibitor cocktail; Roche Molecular Chemicals). Immunoprecipitation and immunoblotting were performed as described previously (2). Total cell lysates (for ERK, p38, SAPK, PKB, and PLCγ1 determinations) or immunoprecipitates (for FcεRIγ chain detection) were subjected to SDS-PAGE and immunoblotted using standard procedures. Phospho-specific Abs against ERK1/ERK2 (T202/Y204), p38 (T180/Y182), SAPK/JNK (T183/Y185), Akt/PKB (S473), and PLCγ1 (Y783), and Abs against total PKB, SAPK/JNK, p38, and PLCγ1, were all obtained from Cell Signaling. Antibodies used for immunoprecipitation and immunoblotting were polyclonal anti-FcεRIγ chain and polyclonal anti-LAT (both obtained from Upstate Biotechnology), and antiphosphotyrosine PY99 (Santa Cruz Biotechnology, Inc.). Horseradish peroxidase–conjugated secondary Abs used were as follows: goat anti–mouse IgG (BD Biosciences), donkey anti–rabbit IgG (Amersham Biosciences), and donkey anti–goat IgG (Santa Cruz Biotechnology, Inc.). Relative quantitation of immunoblots was performed using Dolphin-1D software (Kurabo). The relative phosphorylation of each signaling protein was normalized to its protein level in each sample.

REFERENCES

1. Roberts, A.W., C. Kim, L. Zhen, J.B. Lowe, R. Kapur, B. Petryniak, A. Spaetti, J.D. Pollock, J.B. Borneo, G.B. Bradford, S.J. Atkinson, M.C. Dinauer, and D.A. Williams. 1999. Deficiency of the hematopoietic cell-specific Rho family GTPase Rac2 is characterized by abnormalities in neutrophil function and host defense. *Immunity*. 10:183–196.
2. Irie-Sasaki, J., T. Sasaki, W. Matsumoto, A. Opavsky, M. Cheng, G. Welstead, E. Griffiths, C. Krawczyk, C.D. Richardson, K. Aitken, et al. 2001. CD45 is a JAK phosphatase and negatively regulates cytokine receptor signalling. *Nature*. 409:349–354.