Sakata et al., http://www.jem.org/cgi/content/full/jem.20062647/DC1

SUPPLEMENTAL MATRIALS AND METHODS

Generation and breeding of mDia1-deficient mice. The mDia1-floxed mutant (available from the eXintegrator system [http://www.cdb.riken.go.jp/scb/MartinG/html/exintegrator.html#] under accession no. CDB0437K) was established as follows. A genomic clone containing the mouse mDia1 gene was isolated from the RP23-407E2 mouse female (C57BL/6J) bacterial artificial chromosome library (BACPAC Resources Center). The loxP-frt/Neo cassette in pBSK (Fig. S1 A) was generated by modification of the frt/pgk-Neo-pA/frt in Bluescript (1) and the loxP/PGK-Neo-pA/loxP, pMCDT-A cassette (available at http://www.cdb.riken.go.jp/arg/cassette.html) (2), a gift from Shin-ichi Aizawa (RIKEN Kobe Institute, Kobe, Japan). A 4-kbp 5′ fragment (chromosome 18, nt 35066636–35062572) to exon 1 was inserted into multiple cloning site (MCS) 1 in the above cassette as the short arm. A 3.1-kbp fragment containing exon 1 (chromosome 18, nt 35062571–35059388) was ligated in a Hind3 site. A 5.8-kbp 3′ fragment to exon 1 (chromosome 18, nt 35059387–35053557) was inserted into MCS2 as the long arm. The targeting vector was linearized with Asc1 and introduced into TT2 embryonic stem cells by electroporation. Cell clones resistant to G418 were isolated, and homologous recombination was examined with PCR and Southern blotting (3). The positive embryonic stem cell clones were microinjected into eight cell-stage embryos derived from ICR strain mice, and chimeric offspring were generated (2). Chimeras were bred with C57BL/6N female mice twice, and resultant heterozygotes were crossed with EIIa-Cre mice (The Jackson Laboratory) to produce heterozygous mutants ($mDia1^{+/-}$) lacking exon 1 and the neomycin gene. $mDia1^{+/-}$ heterozygous mice lacking the Cre gene were obtained by mating with C57BL/6N mice again and were intercrossed to produce $mDia1^{-/-}$ homozygotes. Genotyping analysis was performed by PCR using the genomic DNA from mice tails. PCR primer pairs were F1 (5′-CTCCAGTCGGGCATGGTAACTGTG-3′) and R1 (5′-GTTCAGCTCCACCCTCTGTACAGC-3′) for examining the exon 1 deletion, or Cre1 (5′-GCGGTCTGGCAGTAAAAACTATC-3′) and Cre2 (5′-GTGAAACAGCATTGCTGTCACTT-3′) for detection of the Cre gene. Loss of mDia1 protein was examined by Western blotting. Systemic histological analysis was performed on 8–9-wk-old wild-type and $mDia1^{-/-}$ male and female mice ($n = 3$ each) using hematoxylin-eosin staining. Organs examined included the brain, pituitary, liver, pancreas, kidney, spleen, thymus, heart, lung, adrenal gland, the gastrointestinal tract, mesenteric lymph node, testis, bladder, skeletal muscle, blood vessel, and bone (sternum and femur).

RT-PCR analysis of WASP mRNA. Total RNA was isolated by using an RNeasy mini kit (QIAGEN), according to the manufacturer's instructions. RT-PCR was performed on 500 ng of total RNA using SuperScript one-step RT-PCR with Platinum Taq (Invitrogen) with a set of WASP primers (5′-AGAGGAGGGCTCCCACCTGTG-3′ and 5′-GGTGCTCCGATATCAGCTTTG-3′, yielding a 211-bp amplified fragment) and, as a positive control, a set of hypoxanthine-guanine phosphoribosyltransferase primers (5′-GCTGGTGAAAAGGACCTCT-3′ and 5′-CACAGGACTAGAACACCTGC-3′, yielding a 250-bp amplified fragment). cDNA synthesis and predenaturation conditions were as follows: 30 min at 50°C and 2 min at 94°C. PCR amplification conditions were as follows: 15 s at 94°C, 30 s at 57°C, and 1 min at 72°C for 35 cycles; and 10 min at 72°C as a final extension. For real-time PCR, complementary DNA was synthesized using SuperScript (Invitrogen). The amount of mRNA for WASP and GAPDH was quantified by a real-time PCR system (LightCycler 2.0; Roche Diagnostics). The primer sequences used were as follows: 5′-CACCACCAGCACCAATCA-3′ and 5′-CCTAGAGATAGTGGACCACCTGA-3′ as the forward and reverse primers, respectively, for WASP, and 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ as the forward and reverse primers, respectively, for GAPDH. Data were analyzed by LightCycler Probe Design software (version 4.0; Roche Diagnostics).

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