SUPPLEMENTAL MATERIALS AND METHODS

Cell Culture and Transfection

Human embryonic kidney cell line HEK293T, HEK293, Hela and RAW264.7 cells were cultured in Dulbecco's modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria), 10 units/ml penicillin-G, and 10 mg/ml streptomycin. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. HEK293T cells were transfected using calcium phosphtate precipitation. HEK293 cells and Hela cells were transfected with Lipofectamine 2000 reagent (Invitrogen).

Luciferase Reporter Assay

293T cells were plated at a subconfluent density and co-transfected with 0.5μg of expression vectors, 0.05μg of the reporter plasmid, and 0.02μg of Renilla luciferase pRL-TK as an internal control for transfection efficiency. Cell lysates were prepared 24h after transfection and the reporter activity was measured using the Dual-luciferase reporter assay system (Promega). Transfections were performed in triplicate and repeated three times to ensure reproducibility.

Plasmid Construction

To construct the RACK1 expression vector, the open reading frame of

human RACK1 cDNA was cloned into the eukaryotic expression vector pCMV-myc and fused to a COOH-terminal myc. The open reading frame of human TRAF2 cDNA was cloned into the eukaryotic expression vector pCMV-Tag2B (Invitrogen). For purifying GST-RACK1 protein, the open reading frame of human RACK1 cDNA was cloned into the expression vector pGEX-4T-1.

RNA extraction and Real Time PCR (RT-PCR) analysis

Total RNA was isolated from 293T cells and macrophages by using TRIzol reagent (Invitrogen). 2μg of total RNA with high quality was processed directly to cDNA with the reverse transcription kit (Promega, Madison, WI), following the manufacturer's instructions, in a total volume of 25μl. RT-PCR reactions were performed in a 20μl volume of the LightCycler-DNA Master SYBR Green I mixture from Roche Applied Science as follows: with 10pmol of primer, 2mM MgCl2, 200μM dNTP mixture, 0.5units of *Taq* DNA polymerase, and universal buffer. All of the reactions were performed in triplicate in an iCycler iQ System (Bio-Rad). To confirm specificity of amplification, the PCR products from each primer pair were subjected to a melting curve analysis and electrophoresis in 2% agarose gel.

Western Blot analysis

After cultured to 80% confluence, cells were lysed and scraped in RIPA buffer and the lysates were centrifuged at 14000rpm (4°C for 15min). Protein concentrations were determined using Bradford reagent (Sigma) according to the Manufacturer's instructions. Equal amounts of total cellular protein were mixed with loading buffer (62.5mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, and bromphenol blue), boiled for 5min, and subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 (TBST) and 5% fat-free dry milk for 1h at room temperature and incubated overnight with primary antibodies in TBST with 1% bovine serum albumin. After washing with TBST, the membranes were further incubated for 1h at room temperature with corresponding horseradish peroxidase-conjugated secondary antibody in appropriate dilution and then washed five times with the same buffer. The immunoreactive protein bands were visualized by ECL kit (Pierce).

Nuclear Protein Extraction

Nuclear extracts were prepared by the mini-extraction method. Cells were washed with ice-cold PBS and harvested by being scraped in 1.5ml PBS followed by pelleting and resuspending in 400μl HEPES/potassium hydroxide (10mM), pH 7.9, 1mM dithiothreitol, 0.1mM EGTA, 0.1mM

EDTA, 0.5mM phenylmethylsulfonylfluoride, and 0.2mM phenylmethylsulfonyl fluoride buffer. The resuspended cells were lysed in 0.5% Nonidet P-40 for 30min after vortexing for 1min, followed by centrifugation at 1,000g for 5min to pellet the nuclei. After separation of the cytoplasmic fraction, nuclei were harvested by resuspension in ice-cold buffer containing 20mM HEPES/potassium hydroxide, pH 7.9, 0.4mM sodium chloride, 1mM dithiothreitol, and 0.2mM phenylmethylsulfonyl fluoride. Tubes were incubated for 1h on ice and then centrifuged to clear the cellular debris. Nuclear extracts were immediately used for Western blot.

RNAi-mediated Knockdown of RACK1

In our experiments, FG12 lentiviral vector, which has an independent open reading frame of green fluorescence protein (GFP), was used to produce small, double-stranded RNA (siRNA) to inhibit target gene expression in 293T cells. To construct the hairpin siRNA expression cassette, complementary DNA oligonucleotides for siRNA of RACK1 (si RACK1) or mutated sequence of RACK1 siRNA as control (si con) were synthesized, annealed, and inserted into FG12. Two human RACK1 siRNA constructs were used as follows: RACK1 siRNA 1# 5’-ACCATCATCATGTGGAAA-3’; RACK1 siRNA 2# 5’-GCAAACACCTTTACACGC-3’; siRNA con vector

5’-GTACATAGGGACGTAACG-3’.

The oligo for mouse RACK1 RNAi were synthesized as the following sequence:5’-GGATGAGAGTCATTCAGAATG-3’. And the control sequence is 5’-GTTCTCCGAACGTGTCACGTTT-3’.

Immunoprecipitation

Cells were washed with ice-cold PBS and lysed in Tris-buffered saline (pH 7.4), containing 50mM Tris, 150mM NaCl, 1% NP-40, 1mM EDTA, 1mM Na3VO4, 10mM NaF, 2.5mg/ml aprotinin and leupeptin, 1mM β-glycerophosphate and AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), and 10mM iodoacetate. Lysates were incubated on ice for 15min before cellular debris and nuclei were removed by centrifugation at 10000g for 5min. Cell lysates were incubated with the corresponding primary antibody overnight at 4°C. Protein A-Sepharose (Amersham Biosciences, Piscataway, NJ, USA) beads in a 50:50 mixture in 50mM Tris buffer, pH 7.0, were added, and further incubated for another 4h at 4°C. The immunoprecipitates were washed four times in Tris-buffered saline and boiled for 5min in 40μl Laemmli buffer containing 0.02% blue bromophenol and 2% bmercaptoethanol.

Macrophage preparation.

Mice were injected intraperitoneally with 1.5 ml of 3% (wt/vol) thioglycolate (BD Bioscience). Peritoneal exudates were isolated 3 d after

injection by lavage of the peritoneal cavity with 10 ml ice-cold PBS solution. Cells were then plated in cell culture plates, were washed twice with PBS solution 2 h after plating and were incubated overnight in DMEM with 10% (vol/vol) FBS before experiments.