Supplementary material

for manuscript Nº 80885 : RIP4 (DIK/PKK), a novel member of the RIP kinase family, activates NF-kB and is processed during apoptosis.

Additional Material and Methods

Reagents

Recombinant FasL, enhancer M2, recombinant TNF, IL-1□ and zVAD were from Apotech (San Diego).

Apoptosis induction

Apoptosis of 293T cells was induced for 4 hours by 100ng/ml recombinant FasL + 1□□g/ml enhancer M2, 24 hours after transfection. Inhibition of apoptosis was achieved by adding 50□□M zVAD prior to stimulation with FasL. Pellets were lysed in 50□mM Tris-HCl pH□6.8, 10□% glycerol, 2□% SDS, 6□M urea for 5 minutes at RT and lysates were sonicated before boiling in reduced SDS-PAGE sample buffer.

Cell lines and culture conditions

293T and Hela cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FCS and penicillin/streptomycin (100 μg/ml of each).

Expression vectors

The following expression plasmids were kindly obtained from the indicated sources: FLAG-JNK from C. Widmann (Lausanne, Switzerland), HA-p38 from J.S. Gutkind

(NIDR, MD, USA), HA-ERK from J. Pouysségur (Nice, France), v-19 ras from E. Reichmann (Lausanne, Switzerland), FLAG-tagged TRAF3-6 from V. Dixit (San Diego, USA), YFP-IκB, GFP-TRAF6-DN and GFP-TRAF2-DN from H. Wajant (Stuttgart, Germany), VSV-DN-IKKβ from S. Whiteside and A. Israel, (Paris, France), and NF-κBLuc reporter plasmid and myc-IκBα-DN from V. Jongeneel (Lausanne, Switzerland). Renilla-luciferase transfection efficiency vector (phRLTK) was purchased from Promega. DN-constructs of TRAF1 (lacking aa 1-163), TRAF3 (lacking aa 1-297) and TRAF5 (lacking aa 1- 153) were generated by standard PCR amplification and subcloning into a pCR3-derived vector, in frame with an N-terminal FLAG-tag.

Luciferase reporter assays

Cells were co-transfected with 500 ng pNF-κBluc, 20 ng phRLTK, the indicated constructs, and mock plasmid to normalize for the total quantity of transfected DNA. 24 h after transfection, cells were either harvested or, in the case of TNF or IL-1α stimulation, incubated for additional 6h with or without recombinant human TNF or IL-1α (100ng/ml). Cells were lysed and dual luciferase activity was measured in a TD-20/20 luminometer (Turner Designs) using Dual-Luciferase Reporter Assay System (Promega), according to the manufacturers' instructions.