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Supplemental Data

JNK1-Mediated Phosphorylation of Bcl-2 Regulates Starvation-Induced Autophagy

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmid constructions

Myc-Bcl-2 and Myc-vBcl-2 plasmids were created by cloning Bcl-2 and v-Bcl-2 cDNA into pCMV5-Myc1. Bcl-2 S70A, S70E, S87A, T69A/S87A, and T69AS70AS87A (AAA) and T69ES70ES87E (EEE) mutants were created by two-step PCR mutagenesis and then cloned in frame into the pCMV5-Myc1 vector. vBcl-2/cBcl-2 chimeras were created by replacing aa24-34 of the vBcl-2 loop with aa25 to aa95 of the non-structured loop of wild-type and mutant cBcl-2 and the resulting constructs were cloned into pCMV5-Myc1.

Antibodies

Beclin 1, JNK1, p62, Myc, and Bcl-2 were detected using a polyclonal goat or rabbit anti-Beclin 1 antibody (1:500), a goat anti-JNK1 antibody (1:200), a rabbit anti-p62/SQSTM1 antibody (1:100) and monoclonal anti-Myc (1:500) and Bcl-2 (1:100) antibodies (Santa Cruz Biotech Inc.). JNK2, phosphorylated Bcl-2, and phosphorylated JNK were detected using a rabbit polyclonal anti-JNK2 antibody (1:400), a phospho-specific Ser70 rabbit mononclonal ant-Bcl-2 antibody (5H2) antibody (1:200) and a phospho-specific (Thr183/Tyr185) polyclonal rabbit anti-JNK antibody (1:100),

respectively (Cell Signaling Technology). The phospho-specific anti-JNK antibody recognizes both phospho-JNK1 and phospho-JNK2. Flag-epitope was detected using a monoclonal anti-FLAG® M2 antibody (1:1000) (Sigma-Aldrich).

Cell Transfection

Cell transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (InVitrogen). For autophagy assays, Bcl-2 (and Bcl-2 mutants) or MKK7-JNK1 (and MKK7-JNK1 (APF)) were cotransfected with GFP-LC3 at a molecular ratio of 3:1. In triple transfection assays with Bcl-2 plasmids, MKK7-JNK1 and GFP-LC3, the molecular ratio was 2:1:1. For co-IP assays, MKK7-JNK1 or MKK7-JNK1 (APF) was cotransfected with Bcl-2 at a molecular ratio of 5:1. For subcellular fractionation assays, WT Bcl-2 MEFs were transfected with either empty vector or Flag-MKK1-JNK1 constructs.

Metabolic Labeling and Phosphorylation Detection

Transfected cells were cultured overnight in labeling medium (phosphate-free DMEM/10% dialyzed FBS) and then cultured in starvation medium (HBSS minus phosphate) or labeling medium for 4 hours containing 2mCi/ml ^{32}P-orthophosphate. Cells were then lysed in lysis buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, Proteinase Inhibitors Cocktail (Roche Applied Sciences,) and Halt Phosphatase Inhibitor Cocktail (Pierce). Endogenous Bcl-2 or Myc-Bcl-2 was immunoprecipitated from cell lysates with a monoclonal anti-Bcl-2 or monoclonal anti-Myc antibody, respectively; separated by SDS-PAGE; and visualized by autoradiography.

Western Blot Analyses

Cells were lysed in lysis buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, Proteinase Inhibitors Cocktail (Roche Applied Sciences,) and Halt Phosphatase Inhibitor Cocktail (Pierce) on ice for 1 hour, and cellular debris was removed by centrifugation at 14,000 *g*. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a PDGF membrane. After blocking the membrane with 5% milk in Tris-Buffered Saline Tween-20 (TBST) for 1 hour, the membrane was incubated with primary and secondary antibodies for 1 hour each and developed with SuperSignal® West Pico Chemiluminescent Substrate (Pierce).

Co-immunoprecipitation Assays

Beclin 1/Bcl-2 co-immunoprecipitations were performed in MCF7.*beclin 1*, HeLa or MEF cells by lysing cells with lysis buffer (50mM Tris pH 7.9, 150 mM NaCl, 1mM EDTA, 1% Triton-X100, Halt Phosphatase Inhibitor Cocktail, (Pierce) and Protease Inhibitor Cocktail (Roche Applied Sciences)) on ice for 1 hour. To immunoprecipitate endogenous Beclin 1 and endogenous Bcl-2 in MEF cells, immunoprecipitation was performed with a monoclonal anti-Bcl-2 antibody pre-conjugated to agarose (1:20 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoprecipitates were subjected to SDS-PAGE gel electrophoresis and Beclin 1 was detected by immunoblot analysis as described above. To immunoprecipitate endogenous Beclin 1 and Myc-Bcl-2, flag-KSHV v-Bcl-2, Myc-vBcl-2 chimeras in Hela cells and MCF7.*beclin 1* cells, immunoprecipitation of Beclin 1 was performed overnight at 4^0C with a polyclonal goat anti-Beclin 1 antibody (1:100 dilution, Santa Cruz Biotechnology Inc., Santa Cruz, CA).

Immunoprecipitates were subjected to SDS-PAGE and Myc-Bcl-2, flag-KSHV v-Bcl-2, Myc-vBcl-2 chimeras were detected by immunoblot analysis as described above.

Subcellular Fractionation

Subcellular fractionation was performed on 10^9 WT Bcl-2 MEF cells transfected with empty vector or Flag-MKK7-JNK1 constructs. Twenty-four hours after transfection, cells were either starved in HBSS or grown in regular growth medium for 4 hours. Cells were then washed 1x with PBS, suspended in fractionation buffer (20 mM Hepes PH7.5, 250 mM sucrose, 1 mM EGTA), and disrupted using a polytron cell homogenizer (Kinematica). After a crude fraction was removed at 700 *g*, a heavy membrane fraction was removed at 7000 *g* for further purification with a Qproteome Mitochondria Isolation Kit **(**QIAGEN in USA). The light membrane fraction was separated from the cytosolic fraction by centrifugation at 280,000 *g*. Proteins were solubilized in lysis buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, Proteinase Inhibitors Cocktail (Roche Applied Sciences,) and Halt Phosphatase Inhibitor Cocktail (Pierce) for Western blot and immunoprecipitation assays.