Silencing an Inhibitor Unleashes a Cytotoxic Enzyme

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EXPERIMENTAL PROCEDURES

Materials. K-562, HeLa, and Hep-3b cells were obtained from the American Type Culture Collection (Manasas, VA). Transfection reagents, cell culture medium, and supplements were from Invitrogen (Carlsbad, CA). GeneEraser™ shRNA Mammalian Expression Vector System was purchased from Stratagene (La Jolla, CA). Oligonucleotides encoding shRNAs were synthesized by Integrated DNA Technologies (Coralville, IA). Enzymes for the manipulation of recombinant DNA were from Promega (Madison, WI).

Chicken IgY antibodies to human RI and goat anti-chicken secondary antibodies were produced by Genetel (Madison, WI). Rabbit anti-actin primary antibody and goat anti-rabbit secondary antibody were from Santa Cruz Biotech (Santa Cruz, CA). Other immunoblotting reagents including HyBond ECL nitrocellulose membrane, ECL detection reagents, and ECL film were from Amersham Biosciences (Piscataway, NJ). [*methyl*-^{3}H]Thymidine was from Perkin Elmer Life Sciences (Boston, MA). All other chemicals and biochemicals were of reagent grade or better and were used without further purification.

Analytical Instruments. Ultraviolet and visible absorption was measured with a Cary Model 50 spectrophotometer (Varian; Sugarland, TX). For assays of cytotoxicity, cells were harvested with a PHD Cell Harvester (Cambridge Technology; Watertown, MA). Radioactivity was measured with a Microbeta Trilux 2 Detector System (Perkin Elmer; Boston, MA).

Creation of shRNA Vectors. A short hairpin RNA (shRNA) was designed to target a vulnerable sequence in RI (*1*). Briefly, oligonucleotides 5′-GAT CCC GGT CCT GTC CAG CAC ACT ACG AAG CTT GGT AGT GTG CTG GAC AGG ACC TTT TTT-3′ and 5′-CTA GAA AAA AAG GTC CTG TCC AGC ACA CTA CCA AGC TTC GTA GTG TGC TGG ACA GGA CCG GG-3′ were inserted into the *Bam*HI and *Xba*I sites of plasmid pGE-1. To do so, each oligonucleotide (1 μg) was diluted in 50 μL of 10 mM Tris–HCl buffer, pH 8.0, containing NaCl (50 mM) and EDTA (0.1 mM). The oligonucleotide mixture was heated to 93 °C for 3 min, and then cooled slowly to room temperature. The duplex DNA was inserted into the *Bam*HI and *Xba*I sites of pGE-1 using LigaFast (Promega, Madison, WI) as directed by the manufacturer. A plasmid containing an insert was verified by restriction digest and sequence analysis, and named "pGE-pos". The negative control, plasmid pGE-neg (Stratagene), directs the expression of a scrambled shRNA that does not have significant similarity to sequences in the human genome.

Cell Culture. Hep-3b cells were grown in MEM with Earle's salts, L-glutamine (2 mM), non-essential amino acids (0.1 mM), and sodium pyruvate (1 mM). K-562 cells were grown in RPMI medium 1640 and HeLa cells were grown in DMEM. All culture medium was supplemented with fetal bovine serum (10% v/v), penicillin (100 U/mL), and streptomycin (100 μg/mL). Cultures were maintained in a humidified incubator at 37 °C containing 5% CO_2(g).

Transfection of Human Cells. Transfection of K-562, HeLa, and Hep-3b cells was carried out in 6-well dishes using Lipofectamine 2000 and Opti-MEM medium (Invitrogen, Carlsbad, CA). Briefly, cells were seeded at 0.5×10^6 cells/well and incubated at 37 °C for 6–12 h in normal growth medium. DNA-Lipofectamine 2000 complexes were formed with DNA (4–7 μg) and Lipofectamine 2000 (4–10 μL) as directed by the manufacturer. Cells were washed with warm serum-free Opti-MEM prior to adding DNA–lipid complexes. Cells were incubated with DNA–lipid complexes for 4–6 h, after which the medium was replaced with Opti-MEM containing FBS (10% v/v). Cells were incubated for 44 h before passage to 75 cm^2 flasks containing Geneticin (600 μg/mL). Stably transfected cells were maintained and propagated in normal growth medium containing Geneticin (600 μg/mL).

Production of Ribonucleases. All ribonucleases used in this study were purified as describe previously (*2, 3*). Ribonuclease concentrations were determined by UV spectroscopy using $\varepsilon = 0.72\ (mg/mL)^{-1}cm^{-1}$ at 280 nm for RNase A and its G88R variant, and $\varepsilon = 0.87\ (mg/mL)^{-1}cm^{-1}$ at 280 nm for ONC. Prior to cytotoxicity assays, ribonucleases were dialyzed extensively versus phosphate buffered saline (PBS).

Assays of Cytotoxicity. The cytotoxicity of ribonucleases was determined by measuring the incorporation of [*methyl*-^{3}H]thymidine into the DNA of stably transfected human cell lines. Briefly, cells were seeded in 96-well plate in 100 μL of normal growth medium at a density of 5000 cells/well. Ribonucleases in PBS (5 μL) were incubated with cells for 44 h at 37 °C, followed by incubation with [*methyl*-^{3}H]thymidine (0.4 μCi/well) for 4 h. Cells were harvested onto a glass fiber filter, and the ^{3}H incorporated into each sample was quantitated by scintillation counting. Cells incubated with PBS alone were used to determine 100% ^{3}H incorporation. IC_{50} values were determined by fitting the data to the equation: $S = 100 \times IC_{50}/(IC_{50} + [\text{ribonuclease}])$, where S is the percentage of [*methyl*-^{3}H]thymidine incorporation after a 48-h incubation with a ribonuclease.

Immunoblotting. The soluble protein fraction of stably transfected cells was prepared from a wet cell pellet using MPER containing HALT protease inhibitor cocktail (Pierce Biotech, Rockford, IL) Briefly, cells from a 75-cm^2 culture flask were harvested and washed with PBS (3×). The cell pellet was resuspended in MPER (10 μL/10^6 cells) and incubated on ice for 30 min. Cells were passed (5×) through a 24-gauge syringe, and cell debris was collected by centrifugation at 15,000g for 10 min. The total protein concentration of cells lysates was determined with a BCA assay (Pierce Biotechnology, Rockford, IL) using bovine serum albumin (2.5–40 μg) as a standard. The total protein concentration of cell lysates ranged from 5.5–16 mg/ml, and varied by <10% between pGE-pos and pGE-neg samples for a given cell line. Cell lysates were stored at –80 °C until use.

Lysates (30 μg of total protein) from stably transfected cells were subjected to SDS–PAGE on a 4–15% w/v Tris–HCl Ready Gel (BioRad, Hercules, CA) including recombinant human RI (1–100 ng) and Precision Plus prestained MW standards (BioRad, Hercules, CA) as standards. Proteins were transferred to HyBond ECL nitrocellulose and then blocked overnight in TBS-T [20 mM Tris, pH 7.5, containing NaCl (0.137 mM) and Tween 20 (0.2% w/v)] containing non-fat dry milk (4% w/v). Blots were incubated with anti-hRI IgY (1:3000 dilution in blocking solution) for 1 h. and then washed with TBS-T (4 × 15 mL). Blots were then incubated with an anti-chicken antibody conjugated to horseradish peroxidase (1:5000 dilution in blocking solution) for 1 h, washed with TBS-T (4 × 15 mL). RI was visualized using ECL detection reagents and exposure to ECL film. Bands were analyzed from a scanned film image with the program ImageQuantTL (Sunnyvale, CA).

To ensure equal loading of cell lysate samples, the blot was stripped and re-probed for actin. Briefly, blots were incubated with 30 mL Restore Western Blot Stripping Buffer (Pierce Biotechnology, Rockford, IL) for 30 min and then washed briefly with TBS-T (20 mL). Blots were then incubated with primary and secondary antibodies, visualized, and analyzed as described above. Rabbit anti-actin and HRP-conjugated goat anti rabbit antibodies were diluted 1:5000 and 1:10,000, respectively, in blocking solution.

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