Knockout of the Ribonuclease Inhibitor Gene Leaves Human Cells Vulnerable to Secretory Ribonucleases

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EXPERIMENTAL PROCEDURES

Materials. Reagents were from Sigma Chemical (St. Louis, MO), Invitrogen (Carlsbad, CA), or VWR (Radnor, PA), and were used without further purification. Antibodies to RNH1 and vinculin, as well as goat anti-mouse IgG–HRP secondary antibody, were from Santa Cruz Biotechnologies (Santa Cruz, CA). Hydrogen peroxide was used within a month of its receipt. Diethyl maleate was from Santa Cruz Biotechnologies. QBI-139 was a kind gift from Dr. L. E. Strong (Quintessence Biosciences, Inc., Madison, WI). Aqueous solutions were made with water that was generated with an Atrium Pro water purification system from Sartorius (Bohemia, NY) and had resistivity ≥18 MΩ $\cdot$cm^{-1}.

HeLa Cell Culture. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing fetal bovine serum (FBS) (10% v/v) and penicillin–streptomycin solution (1% v/v). Cells were incubated at 37 °C in a humidified incubator under 5% v/v CO_2(g).

Generation of *RNH1*–Knock-Out HeLa Cells. Seven sets of small guide RNA mapping to exons 1 and 2 of *RNH1* were created using the CRIPSR RGEN Tools available through the Center for Genome Engineering, Institute for Basic Science, Seoul, South Korea (www.rgenome.net). sgRNA and SpCas9 plasmids were transfected into 1×10^5 cells with Lipofectamine 2000 from Invitrogen. To check the activity of sgRNA in HeLa cells, genomic DNA was isolated after 48 h with a DNeasy Blood & Tissue kit from Qiagen (Hilden, Germany), and the target site was amplified for deep sequencing. Deep sequencing libraries were generated by PCR, and TruSeq HT Dual Index primers from Illumina (San Diego, CA) were used to label each sample. Pooled libraries were subjected to paired-end sequencing at LAS (Seoul, South Korea). Indel frequencies were calculated as described previously.[1] To obtain single clones, cells were diluted in a 96-well plate at a limiting dilution with a 0.3 cell/well average. After 15 to 21 days in culture, 45 clones were grown to confluency. A small portion of these cells were maintained in culture and the rest were used for the isolation of genomic DNA, which was then subjected to targeted deep-sequencing analysis to validate the knock-out. Of the 45 clones tested, 39 were *RNH1* knock-outs. The clone used in this study was chosen based on deep-sequencing results as well as immunoblot analysis (*vide infra*). This clone has a deletion in nucleotides 6038–6042 on exon 2, causing a frameshift mutation after Glu68.

Immunoblotting. Cells were seeded into 6-well plates and grown to 80-100% confluency, then lysed using M-PER™ Mammalian Protein Extraction Reagent from ThermoFisher (Waltham, MA) with the addition of Pierce™ Protease Inhibitor Mini Tablets from ThermoFisher. Cell lysates were spun down for 7 min at 14,000*g* to remove cell debris, and the total protein concentration in the supernatant was determined with a BCA Assay kit from ThermoFisher. Protein (65 μg) was then loaded onto a 10% w/v mini-PROTEAN® TGX™ SDS–PAGE gel from Biorad (Hercules, CA) along with recombinant RNH1 and a MagicMark™ immunoblot ladder from Invitrogen. Recombinant RNH1 was purified as described previously,[2] and was a kind gift from Dr. T. T. Hoang (University of Wisconsin–Madison). The gel was subjected to electrophoresis at 70 V for 1 h, and protein was transferred to a PVDF membrane with an iBlot 2 dry transfer system from Invitrogen. The gel was then cut at the 100-kDa mark to allow simultaneous incubation of antibodies to RNH1 and vinculin. The two halves were blocked for 1 h in a solution of dry milk (5% w/v) in TBS-Tween (TBS-T), washed, and then incubated overnight at 4 °C with their respective antibody (1:1000 dilution) in TBS-T containing BSA (5% w/v). After another wash with TBS-T, membranes were incubated with secondary antibody (1:5000 dilution), washed again, and then imaged with an Amersham ECL Select Western

Blotting Detection Reagent and an ImageQuant LAS4000 instrument from GE Healthcare (Chicago, IL).

Assays of Cell Proliferation. Cell proliferation was measured by using the CyQUANT® NF Cell Proliferation Assay from ThermoFisher. Briefly, cells were grown to 80–100% confluency in DMEM containing FBS (10% v/v) and penicillin–streptomycin solution (1% v/v), and then counted with a Coulter counter. The medium was replaced with DMEM containing penicillin–streptomycin solution (1% v/v) but not FBS, and seeded into the inner wells of a black 96-well plate at 500 cells per well. After 24 h (plotted as $t = 0$), medium in the first 4 wells was replaced with 75 μL of CyQUANT dye-mix prepared according to the manufacturer's instructions. After 1 h, fluorescence readings were measured on an Infinite M1000 plate reader from Tecan (Männedorf, Switzerland) with excitation at 485 nm, emission at 530 nm, and optimal gain. Readings of 4 additional wells were then taken at 24-h intervals until cells reached confluency, with fluorescence readings normalized to the initial reading at $t = 0$. For these points, the gain was set manually to the optimal gain determined after the first reading to facilitate comparison between time points. Dye mix was prepared freshly before each reading, and medium was replaced in unused wells every 48 h. All data points are the compilation of at least 3 biological replicates.

Purification of Ribonucleases. RNase 1 and its H12A variant were purified as described previously,[3] with the following exceptions. After induction, cells were lysed with a benchtop cell disrupter from Constant Systems (Kennesaw, GA), which was set to 19.0 kpsi. The lysate was then subjected to centrifugation at 10,500 rpm for 45 min, and inclusion bodies dissolved in 20 mM Tris–HCl buffer, pH 8.0, containing guanidine–HCl (7 M), EDTA (10 mM), and DTT (0.10 M). The resulting solution was diluted 10-fold by the slow addition of a degassed 20 mM acetic acid, and then subjected to centrifugation again at 10,500 rpm for 45 min. The supernatant was dialyzed in 3.5-kDa MWCO tubing from Spectrum Labs (Rancho Dominguez, CA) against 16 L of 20 mM acetic acid overnight. The retentate was subjected to centrifugation at 10,500 rpm for 30 min, added dropwise to folding solution, which was 100 mM Tris–HCl buffer, pH 7.8, containing NaCl (0.10 M), reduced glutathione (1.0 mM), and oxidized glutathione (0.2 mM), and then allowed to fold at 4 °C for 2–5 days. The pH of the solution was adjusted to 5.0, and the solution was concentrated to a final volume of 10 mL using an Amicon® Stirred Cell concentrator from EMD Millipore (Billerica, Massachusetts) with Hydrosart® 10-kDa filters from Sartorius. To purify the protein further, the resulting solution was subjected to chromatography on a HiLoad® 26/600 Superdex® 75 pg gel-filtration column from GE Healthcare (Chicago, IL) with 0.05 M sodium acetate buffer, pH 5.0, containing NaCl (0.10 M) and sodium azide (0.05% w/v). The protein was then run on a HiTrap SP HP cation-exchange column from GE Healthcare before being dialyzed against PBS. Ribonucleolytic activity was measured by using an assay described previously.[4,5]

Assays of Cell Viability. Cell-viability assays were performed with the CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega (Madison, WI), which is a tetrazolium dye-based assay for cell metabolic activity. In short, cells were grown to 80–100% confluency in DMEM containing FBS (10% v/v) and penicillin–streptomycin solution (1% v/v), then counted with a Coulter counter and seeded into the inner wells of a flat-bottomed 96-well plate at 5000 cells per well. The cells were incubated for 24 h, after which the medium was replaced with DMEM containing the various concentrations of a ribonuclease or oxidant. For experiments with oxidants, the medium also contained FBS (10% v/v). For experiments with ribonucleases, the medium was FBS-free medium to minimize any contamination from serum

ribonucleases. (Note: The presence of FBS did not alter the viability significantly in either cell line.) After a 48-h incubation, the medium was replaced again, 20 μL of MTS reagent was added to each well, and the resulting plate was incubated for 1 h. Absorbance at 490 nm was then measured on an Infinite M1000 plate reader, and percent viability was calculated by using the highest and lowest absorbance values. Each plate contained no cell and untreated controls to enable normalization. Data were analyzed with software from GraphPad Prism (La Jolla, CA). Values of EC_{50}, which is the concentration of analyte that gives half-maximal cell viability, were calculated using the equation:

$$y = y_{\min} + \frac{(y_{\max} - y_{\min})}{1 + (\frac{EC_{50}}{x})^h}$$

where y is cell viability, x is the concentration of analyte, and h is the Hill coefficient. To aid visualization, data were plotted on a semilogarithmic scale. All data points are the compilation of at least 3 biological replicates.

Ribonucleolytic Activity Assays on Media. Ribonucleolytic activity was measured using a fluorogenic assay as described previously.[4,5] In short, cells were grown to 80–90% confluency and add to the wells of 6-well plates at a density of 100,000 cells/well. After 24 h, medium was replaced with FBS-free medium, and cells were incubated another 48 h. Medium with and without FBS (10% v/v) was also added to empty 6-well plates and incubated for 48 h to serve as a reference. After 48 h, the different media were collected and assayed immediately for ribonucleolytic activity. To do so, 10 μL of medium was added to 200 μL of 100 mM Tris–HCl buffer, pH 7.4, containing NaCl (100 mM) and the fluorogenic substrate. Activity was also compared with recombinant RNase 1 purified as described above at 0.7 pM concentration. The increase in fluorescence in at least 3 biological replicates was measured over the course of several minutes.

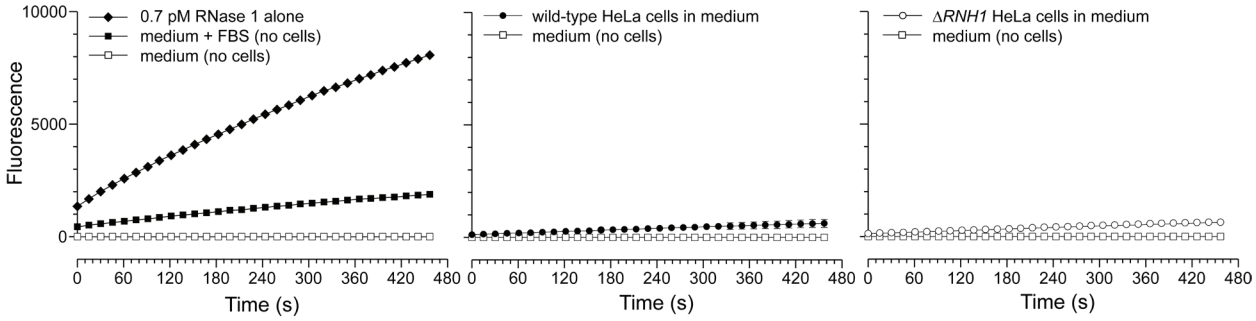

Figure S1: Ribonucleolytic activity of RNase 1 alone, media without cells, and media after the growth of wild-type and Δ*RNH1* HeLa cells. Data are values (±SE) of fluorescence intensity averaged from three biological replicates. The data for medium alone are included in each panel for reference.

REFERENCES

(1) Kim, D., Bae, S., Park, J., Kim, E., Kim, S., Yu, H. R., Hwang, J., Kim, J.-I., and Kim, J.-S. (2015) Digenome-seq: Genome-wide profiling of CRISPR–Cas9 off-target effects in human cells, *Nat. Methods 12*, 237–243.

(2) Johnson, R. J., McCoy, J. G., Bingman, C. A., Phillips, G. N., Jr., and Raines, R. T. (2007) Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein, *J. Mol. Biol. 367*, 434–449.

(3) Rutkoski, T. J., Kurten, E. L., Mitchell, J. C., and Raines, R. T. (2005) Disruption of shape-complementarity markers to create cytotoxic variants of ribonuclease A, *J. Mol. Biol. 354*, 41–54.

(4) Kelemen, B. R., Klink, T. A., Behlke, M. A., Eubanks, S. R., Leland, P. A., and Raines, R. T. (1999) Hypersensitive substrate for ribonucleases, *Nucleic Acids Res. 27*, 3696–3701.

(5) Eller, C. H., Lomax, J. E., and Raines, R. T. (2014) Bovine brain ribonuclease is the functional homolog of human ribonuclease 1, *J. Biol. Chem. 289*, 25996–26006.