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

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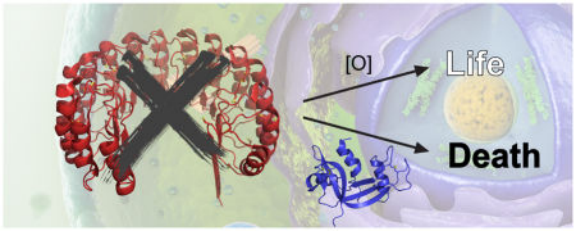
Abstract

Ribonuclease inhibitor (RNH1) is a cytosolic protein that binds with femtomolar affinity to human ribonuclease 1 (RNase 1) and homologous secretory ribonucleases. RNH1 contains 32 cysteine residues and has been implicated as an anti-oxidant. Here, we use CRISPR–Cas9 to knockout *RNH1* in HeLa cells. We find that cellular RNH1 affords marked protection from the lethal ribonucleolytic activity of RNase 1 but not from oxidants. We conclude that RNH1 protects cytosolic RNA from invading ribonucleases.

Graphical Abstract



Ribonuclease inhibitor (RNH1[1,2]) is a highly conserved cytosolic protein of micromolar abundance that binds to human ribonuclease 1 (RNase 1), bovine RNase A,[3] and other pancreatic-type ribonucleases[4] with femtomolar affinity.[5,6] Although many of the physical properties of RNH1 are well-understood, its biological function is subject to debate. Because

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Author Contributions

S.P.T. and R.T.R. designed the experiments. E.K. and J.-S.K. generated *RNH1*–knockout HeLa cells. S.P.T. performed immunoblots, proliferation, and cytotoxicity assays. S.P.T. and R.T.R. wrote the manuscript. All authors have given approval to the final version of the manuscript.

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of its high affinity for pancreatic-type ribonucleases, which are secretory enzymes, RNH1 has been called a cellular “sentry” that protects cytosolic RNA from degradation by endocytosed ribonucleases.[7] This hypothesis is supported by RNase 1 variants with decreased affinity for RNH1 being toxic to human cells, unlike wild-type RNase 1.[6,8,9] Yet, when RNAi has been used to diminish levels of RNH1,[10–15] the results have been used to advance the hypothesis that RNH1 acts as a cytosolic antioxidant.[10,11,14] As RNH1 contains 32 cysteine residues and its concentration in the cytosol is ~4 μM,[7] RNH1 provides ~0.1 mM thiol groups. For comparison, the intracellular concentration of reduced glutathione ranges from ~0.1–15 mM depending on the subcellular compartment, cell-type, and organism.[16]

Distinguishing between these two hypotheses with RNAi is difficult. By mediating gene expression at the level of transcription, RNAi suffers from incomplete and often transient knock-downs, as well as off-target effects.[17] The task with RNH1 is a special challenge. As RNH1 binds to RNase 1 with extraordinary affinity and the entry of RNase 1 into the cytosol is inefficient,[18–20] even a low level of RNH1 could protect a cell.[1] In contrast to RNAi, methods that employ CRISPR–Cas9 act at the genomic level.[21–24] Differences in phenotypes arising from knockdowns by RNAi and knockouts by CRISPR–Cas9 have been reported, as have identical RNAi-mediated knock-downs in different genetic backgrounds.[25–27] Here, we report on a CRISPR–Cas9 knockout of *RNH1* in a human cell, allowing for an unambiguous assessment of its biological role.

We created a CRISPR-edited knockout of *RNH1* in HeLa cells.[28] The most successful knockout clone (Δ*RNH1*) contained a 5-nucleotide deletion in exon 2. Δ*RNH1* cells exhibited no detectable production of RNH1 protein (Figure 1A), but proliferated at a rate indistinguishable from that of wild-type HeLa cells (Figure 1B). These coinciding cell-growth curves from wild-type and knockout cells conflict with results from RNAi-mediated knock-down experiments.[13,14] This concurrence indicates that any leakage of nascent RNase 1 from the secretory pathway of an Δ*RNH1* cell into its cytosol is inconsequential. Lastly, the growth medium from each cell line contained an equivalent (low) amount of ribonucleolytic activity (Figure S1 in the Supporting Information), suggesting that the production of RNase 1 is not linked to that of RNH1.

RNH1 protects HeLa cells from RNase 1. *RNH1*-knockout cells were much more vulnerable to wild-type RNase 1 than were wild-type cells (Figure 2A). Indeed, no deleterious consequences on wild-type cells were apparent with RNase 1 at 17 μM, which is the EC_{50} value for *RNH1*-knockout cells. In contrast, *RNH1*-knockout cells and wild-type cells were equally vulnerable to QBI-139 (Figure 2B), which is an RNase 1 variant that has been engineered to evade RI and is in clinical trials as a cancer chemotherapeutic agent.[29,30] Finally, neither *RNH1*-knockout cells nor wild-type cells are vulnerable to H12A RNase 1 (Figure 2C), which is a variant having a substitution in a key active-site residue and thus low catalytic activity.[31,32] These cell viability data (Table 1) provide strong support for the hypothesis that RNH1 modulates the toxicity of RNase 1 by inhibiting its ribonucleolytic activity.

RNH1 affords little protection from oxidative stress. HeLa cells were subjected to three well-known inducers of oxidative stress—hydrogen peroxide (H_2O_2), diethyl maleate (DEM), and sodium arsenite ($NaAsO_2$). *RNH1*-knockout cells and wild-type cells were equally vulnerable to H_2O_2 and DEM (Figures 2D and 2E). *RNH1*-knockout cells appeared to be slightly more vulnerable to $NaAsO_2$ than were wild-type cells (Figure 2F), but the corresponding ratio of EC_{50} values was only twofold (Table 1). Thus, the marked protection afforded by RNH1 to the potential toxicity of RNase 1 was not replicated with inducers of oxidative stress.

RNase 1 exists in all bodily fluids. Endothelial cells have been shown to secrete up to 100 ng of RNase 1 per 10^6 cells on a daily basis.[33–35] This enzyme can re-enter endothelial cells by endocytosis, suggesting that the cytosol is assaulted constantly by a potent catalyst of RNA degradation.[32,36–38] By binding and inactivating RNase 1,[6] RNH1 can protect cytosolic RNA from degradation and promote cell survival.

The observed EC_{50} value for RNase 1 (17 μM) is much greater than the concentration of RNase 1 in human serum (480 ng/mL = 33 nM).[39] HeLa cells are, however, nearly 10^2-fold less vulnerable to ribonucleases than are other cell lines.[12,40] Moreover, even modest cytotoxicity could be intolerable in a physiological context.

Although the 32 cysteine residues of RNH1 contribute little to cellular oxidation-resistance, they might have evolved for another purpose. RNH1 is sensitive to oxidation, and oxidized RNH1 is incapable of binding to a ribonuclease.[41–43] This sensitivity extends to RNH1·ribonuclease complexes, which release active enzyme upon oxidation.[2,44] All vertebrates have intracellular RNH1. The human protein is, however, more sensitive to oxidation than is that of other mammals, a bird, or a lizard.[2] Together, these data suggest that human RNH1 might have evolved to be a highly sensitive trigger for unleashing lethal ribonucleolytic activity in response to oxidative stress, which would otherwise inflict heritable genomic damage.

The clinical efficacy of an RNase 1 variant, QBI-139, relies on its ability to evade RNH1.[29,30] The viability of HeLa cells is affected by QBI-139 regardless of the presence of RNH1 (Table 1; Figure 2B). Moreover, the viability of HeLa cells that lack RNH1 is affected equivalently by QBI-139 and wild-type RNase 1. These data suggest that QBI-139 is an optimized cytotoxin. Still, enhancing cellular uptake and endosomal escape could render QBI-139 even more effective.[9,18] The *RNH1*-knockout cells described herein provide a powerful means to explore such approaches without confounding effects from inactivation by RNH1. Experiments along these lines are ongoing in our laboratory.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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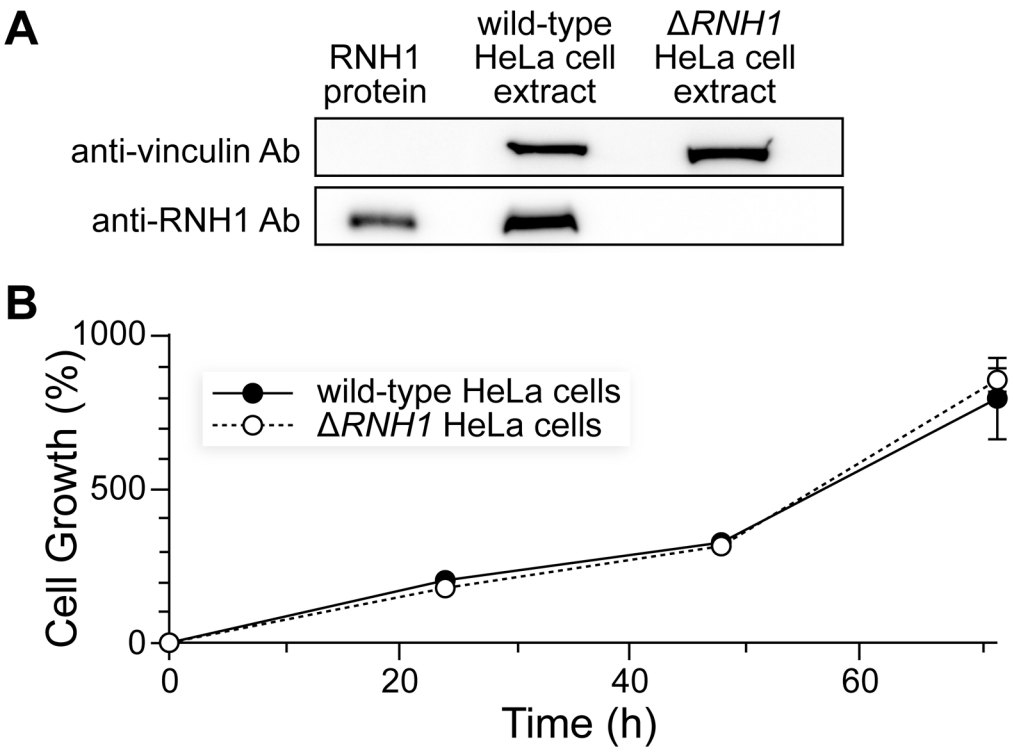

Figure 1.

Characterization of *RNH1*-knockout HeLa cells. (A) Immunoblot of an extract from wild-type and *RNH1* cells. (B) Proliferation of wild-type and *RNH1* cells in serum-free Dulbecco's modified Eagle's medium containing penicillin–streptomycin solution (1% v/v) at 37 °C.

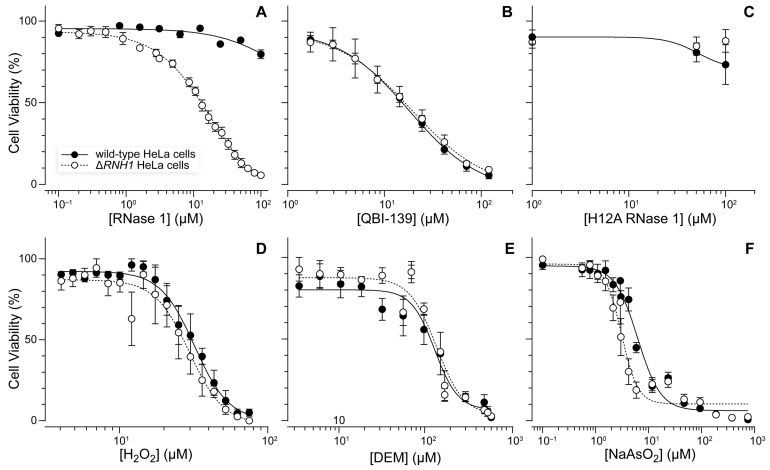

Figure 2.

Effect of human RNase 1 (A), its variants (B, C), and oxidizing agents (D–F) on the viability of wild-type and *RNH1*-knockout HeLa cells. Cell viability was measured with a tetrazolium dye-based assay for metabolic activity. Values of EC_{50} are listed in Table 1.

Table 1

Effect of Human RNase 1, its Variants, and Oxidizing Agents on the Viability of Wild-Type and *RNH1*-Knockout HeLa Cells[a]

	HeLa cell type	
reagent	**wild-type**	**Δ*RNH1***
RNase 1	>100[b]	17 ± 2
QBI-139	18 ± 2	19 ± 6
H12A RNase 1	>100[b]	>100[b]
H2O2	32 ± 6	30 ± 2
DEM	130 ± 17	140 ± 13
NaAsO2	6.2 ± 0.5	3.1 ± 0.2

[a]Data are values (± SE) of EC_{50} (μM) for cell viability as measured with a tetrazolium dye-based assay for metabolic activity.

[b]Protein concentrations >100 μM resulted in >75% cell viability.