Purification and Characterization of Oxidation-Resistant Ribonuclease Inhibitor Variants

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Purification and Characterization of Oxidation-Resistant Ribonuclease Inhibitor Variants

Alec Uebersohn, Class of 2013

Advisor: Kimberly Dickson

A Paper Submitted for Honors at Graduation, Spring Term 2013.
I hereby reaffirm the Lawrence University Honor Code:

_________________________________

Alec W. Uebersohn
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First and foremost, I want to thank my research advisor, Kimberly Dickson, who first proposed this project to me in the fall of 2011. I can’t imagine where I would be or what I would be doing right now if it hadn’t been for the immensely rewarding research opportunity that she has provided me with. I am a much different person and a much different scientist today that I ever was before I began this working on this study and I like to think that I have grown up with it. The work I’ve done on this project has intersected with so many other aspects of my college experience and always for the better. I look forward to my remaining time in lab working with her to advance forward our collective knowledge of biology and the world around us.

I also must acknowledge my academic advisor, Stefan Debbert, and the director of the CTL, Julie Haurykiewicz, for kicking my butt when it needed to be kicked. They both know what kind of student I once was and I’m glad to have been given a second chance to succeed here at Lawrence.

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I am forever in debt to my family, whose unconditional love and support I have relied on like a rock my entire life. The value of this becomes clearer every day.

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Last, but not least, I am grateful to the funding provided to me by the Andrew W. Mellon Foundation, which allowed me to travel to Boston in April of this year to present my findings at the annual meeting of the American Society of Biochemistry and Molecular Biology.
# Table of Contents

## Abstract

## Introduction

<table>
<thead>
<tr>
<th>Characterization and Structure</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interactions with ribonucleases</td>
<td>9</td>
</tr>
<tr>
<td>Evolution of ribonuclease inhibitor, a leucine-rich repeat protein</td>
<td>16</td>
</tr>
<tr>
<td>Cysteine-mediated oxidation sensitivity</td>
<td>24</td>
</tr>
</tbody>
</table>

## Biological Roles of Ribonuclease Inhibitor

| Distribution within the body and within the cell | 25 |
| Role in cell protection against ribonucleases | 26 |
| Role in managing oxidative stress | 30 |
| Ribonuclease inhibitor and cancer | 33 |
| Other biological roles | 35 |

## Investigating RI by Constructing Oxidation-Resistant RI Variants

| Manipulating RI: where and how? | 37 |

## Optimizing Expression and Purification of Recombinant RI

| Difficulty of RI purification | 40 |
| MBP as a solubility enhancer | 42 |
| Chaperones as co-expression partners | 43 |
| Manipulating redox conditions to optimize RI production | 44 |

## Characterizing RI and RI Variants: Activity, Structure, and Stability

| Determining the inhibitory activity and oxidative resistance of RI variants | 45 |
| Measuring thermal stability: Is it hot in here, or is it just me? | 48 |
| Comparing structures using circular dichroism spectroscopy | 49 |

## Materials and Methods

| General Materials | 50 |
| Development of wild-type RI and RI variant expression constructs | 51 |
| Expression and crude purification of RI and RI variants | 54 |
| Digestion of MBP-RI and MBP-RI variant fusion proteins | 57 |
| Isolation and further purification of RI and RI variants | 57 |
| Differential scanning fluorimetry | 58 |
| RNase A inhibition assays using 6-FAM-dArUdAdA-6-TAMRA | 59 |

## Results

| Expression and purification of RI | 60 |
| Thermal stability of RI and RI variants | 65 |
| Inhibitory activity of RI and RI variants | 65 |

## Discussion

| Expression and purification | 66 |
| Thermal stability and inhibitory activity of RI variants | 68 |

## Works Cited

| 72 |
ABSTRACT

Ribonuclease inhibitor (RI) is an intracellular mammalian protein which binds vertebrate-specific ribonucleases; this interaction is one of the tightest non-covalent interactions yet discovered. The biological activity of RI is poorly understood, but it is thought to regulate the biological functions of ribonucleases, which include initiating blood vessel growth, maintaining neuron viability, attacking pathogens, and mediating cell stress responses. RI is also involved in pathways unrelated to ribonucleases, including interactions with Drosha and PTEN, an anti-tumor protein.

One of the defining characteristics of RI is its oxidation sensitivity, a result of its unusually high cysteine content. The oxidation of RI is all-or-none and leads to complete loss of inhibitory activity; this feature is known to be relevant to at least some of its functions within the cell. However, the importance of these cysteines in the structure, inhibitory activity, and oxidation sensitivity of RI is unknown.

Variants of RI have been created in which sets of cysteines in RI have been conservatively replaced with other amino acids. In this study, we sought to recombinantly express and purify both native RI and the RI variants. Once pure, the variants were characterized for structural stability, inhibitory activity, oxidation sensitivity, and structural similarity to wild-type RI. Preliminary data suggest that the replacement of cysteines leads to an increase in structural stability but a decrease in inhibitory activity. This is a sign that tight binding of ribonucleases and vulnerability to oxidation may have co-evolved.
INTRODUCTION

In 1958, Francis Crick first drew out this famous scheme, defining the flow of genetic information within living organisms (1):

![Diagram of DNA, RNA, and Protein]

Figure 1: A sketch from Francis Crick’s personal notes.

If you’ve taken an introductory science class any time within the last 40 years, you might recognize this as molecular biology’s central dogma. This awe-inspiring chemical reaction was first sparked into motion billions of years ago and has been churning on and on ever since. In words, living organisms are able to rewrite the information contained in our DNA into RNA messengers, which can be translated into proteins. Proteins are thought to serve as the grunts of the biological operation, like little robots, filling thousands of varied roles including digesting complex and diverse nutrients (2), transporting oxygen in blood (3), keeping skin strong and healthy (4), and enabling sight, smell, and touch (5) (6) (7). Because of their primary and measurable role in biochemical functions, proteins have been thoroughly researched. DNA has gotten plenty of attention as well; we are taught from a young age the importance of our genes and how they make us who and what we are.

Much like the middle child that it is, RNA has not received the attention it deserves. RNA is not simply a messenger that reports from the information banks of DNA to the protein factories of the ribosomes. This misunderstanding long stood for a
simple reason: DNA and most proteins are considerably more stable than RNA, and thus were the first biological molecules to be purified and studied (8). However, as analytical techniques have improved, we have been able to take a deeper look into the mystery of life. As we do, the significance of RNA is becoming more and more clear. Specialized forms of RNA are ubiquitous. Transfer RNA (tRNA) and ribosomal RNA (rRNA) are vital pieces of the protein assembly line (9). MicroRNAs can directly regulate the expression of genes, and newly discovered circular RNAs add another step of complexity, by regulating the activity of microRNAs (10). Even more intriguing is the existence of RNA molecules with enzymatic activity; these ancient “ribozymes” can be found catalyzing chemical reactions, a job originally thought to be reserved only for proteins (11). Even in RNA’s classical role as the conduit through which genes become proteins, its importance should not be underestimated.

To better appreciate RNA’s significance, allow me to take you back in time to a world that may have existed billions of years ago: the RNA world. RNA is chemically similar to DNA, including a shared ability to encode genetic information. Some viruses exclusively use RNA to store their genetic information (12). RNA also can act like an enzyme, catalyzing reactions. Given this versatility, it is possible that RNA once performed all of the functions that DNA and proteins perform today. The implications of this possibility are vast for our understanding of how life may have begun. And it isn’t so far-fetched! In 2009, the Nobel Prize in Chemistry was awarded to Thomas Steitz ('62), along with Venkatraman Ramakrishnan and Ada Yonath, for their work in elucidating the structure and mechanism of the ribosome, the omnipresent and ancient macromolecular machine that synthesizes proteins from RNA code (13). Their data confirmed that the ribosome’s activity is actually catalyzed not by its protein structure
but by the ribosomal RNA contained within it. Thus, it appears that while DNA and proteins have been stealing the limelight, RNA has been quietly performing some of the most vital functions of life.

The diverse types of RNA within the cell, which are still being revealed, can be regulated and manipulated by proteins known as ribonucleases, which cleave RNA into smaller pieces. Ribonucleases exist in eukaryotes, prokaryotes, and viruses, suggesting an ancient origin and an essential role in life (14) (15) (16). One family of ribonucleases new on the scene, evolutionarily speaking, is known as the vertebrate-specific ribonuclease family. These ribonucleases cleave single-stranded RNA at highly varied rates (17). These RNA-regulating proteins have their own regulator as well, known as ribonuclease inhibitor.

Ribonuclease inhibitor (RI) is a 49.8-kDa protein found in the interior of cells in mammals (18) (19) (20). This protein has a non-globular, horseshoe-like structure (21) (18) (Figure 2). Although different ribonucleases from the vertebrate-specific family are found fulfilling a wide variety of jobs scattered throughout the body (Table 1), RI is able to bind to nearly all of them, rendering the ribonuclease dead in its tracks (22). Although this binding interaction is reversible, it is certainly on any biochemist’s short list of the tightest protein-protein interactions known (23). Yet, RI is a rather fragile protein—even the oxygen levels found in the bloodstream are high enough to denature it (24).
Figure 2: The Ribonuclease Inhibitor “Horseshoe”. 3D ribbon structure of porcine (pig) RI from (A) top-down and (B) front views. C-terminus shown in orange; N-terminus shown in pink. Blue represents A-type repeats; yellow represents B-type repeats. The helical structures on the outside of the “horseshoe” are called α-helices. The arrows on the interior represent β-strands. Image created using PyMOL from PDB #2BNH. Crystal structure resolved by Kobe and coworkers (21).
Ribonuclease inhibitor is more than just a modulator of ribonuclease activity. Without ribonuclease inhibitor present, vertebrate-specific ribonucleases can cause cell death (25). Why, then, is such an important protein so easily denatured? In this study, we will modify the biochemical properties of human ribonuclease inhibitor in order to investigate the relationship between its oxidiation-sensitive nature and its vital role within the cell.

**Characterization and Structure**

*Interactions with ribonucleases*

RI is able to inhibit the activity of a large number of members of the vertebrate-specific ribonuclease family, or RNase A superfamily, of proteins (26). Ribonucleases from this family are relatively small, composed of about 130 amino acids (27). These ribonucleases cleave single-stranded RNA via hydrolysis of the phospho-ester bonds that link together RNA’s backbone (28). RNase A, a prototypical member of this family, catalyzes this reaction almost as quickly as it can come into contact with RNA through diffusion; it is “an enzyme limited by physics rather than chemistry” (29). Two histidines and one lysine make up the active site of vertebrate-specific ribonucleases. Aside from these three catalytic residues and another six to eight cysteines important to structure, ribonucleases are considerably diverse, with sequence identities as low as 20% (27). Even in the face of this divergence, the non-covalent binding interaction between RI and ribonucleases is exceptional, occurring with femtomolar affinity (23). Eight members of this family have been isolated from human tissue; another five may exist according to genetic analyses (Table 1).
<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>Also Known As</th>
<th>Found In</th>
<th>Proposed Functions</th>
<th>Binds to RI?</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase 1</td>
<td>Human Pancreatic RNase</td>
<td>Pancreas, kidneys, stomach, lungs, liver, spleen, endothelial tissue, urine, blood serum</td>
<td>Maintaining serum viscosity (32); activation of immune system (33)</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 2</td>
<td>Eosinophil-derived neurotoxin (EDN)</td>
<td>Eosinophil granules, spleen, liver, kidneys, placenta, urine</td>
<td>Activation of immune system (33); Antiviral protection (34)</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 3</td>
<td>Eosinophil cationic protein (ECP)</td>
<td>Eosinophil granules</td>
<td>Regulating inflammatory responses (35); antiviral/bacterial/helminth protection (36); induction of apoptosis (37)</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 4</td>
<td>---</td>
<td>Pancreas, lung, skeletal muscle, heart, kidneys, and placenta</td>
<td>Angiogenesis, stimulation of neuronal growth, neuroprotective</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 5</td>
<td>Angiogenin (ANG)</td>
<td>Liver, endothelial cells, spinal cord, cancer cells</td>
<td>Angiogenesis (43); rRNA production, cell proliferation and survival (44); extension and survival of motor neurons, neuroprotective (45); cell stress response (46); cancer cell proliferation (47) (48); Protects skin from bacteria (49)</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 6</td>
<td>---</td>
<td>Lungs, heart, brain, placenta, liver, skeletal muscle, kidney, pancreas</td>
<td>Unknown.</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 7</td>
<td>---</td>
<td>Keratinocytes (51); Liver, kidney, skeletal muscle, heart (52)</td>
<td>Protects skin from bacteria (51)</td>
<td>Yes</td>
</tr>
<tr>
<td>RNase 8</td>
<td>---</td>
<td>Placenta (53)</td>
<td>Antimicrobial protection of placenta (54)</td>
<td>Unknown</td>
</tr>
<tr>
<td>RNase 9-13+f</td>
<td>---</td>
<td>testes (27); other locations possible</td>
<td>Not known; suspected to not be ribonucleolytic; may play a role in male reproduction (27)</td>
<td>Unknown</td>
</tr>
<tr>
<td>RNase A</td>
<td>Bovine Pancreatic RNase</td>
<td>Pancreas of bovines (Bos taurus)</td>
<td>Digestion of RNA from stomach microorganisms (55)</td>
<td>Yes</td>
</tr>
<tr>
<td>BS-RNase</td>
<td>Bovine Seminal RNase</td>
<td>Bovine semen (56)</td>
<td>Immunosupression (56)</td>
<td>No³</td>
</tr>
<tr>
<td>Onconase</td>
<td>ONC; Ranpirinase; P-30</td>
<td>oocytes of northern leopard frogs (57)</td>
<td>Cytotoxic in humans; targets tumor cells (57); used in cancer therapy (58)</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 1: A selection of members of the vertebrate-specific ribonuclease family showing their source and functions, if known. Ribonucleases shown are found in humans unless otherwise noted.

²The tissues listed are where the production of these ribonucleases has been detected; however, ribonucleases have signal peptides that allow them to be secreted from cells (27). Therefore, they may be found elsewhere.
³Eosinophil granules are vacuoles found in eosinophil granulocytes, a type of white blood cell, which contain toxic proteins and compounds that are used to attack pathogens. For further reading, see (59).
⁴Helmints are parasitic worms.
⁵Apoptosis is the self-initiated death of a cell.
⁶Angiogenesis is the growth and development of new blood vessels.
⁷Genes were discovered in the human genome that pointed to the existence of these 5 ribonucleases; little is yet known about their activity or function.
⁸BS-RNase is found in nature as a dimer, i.e. it partners with another copy of itself. The monomer form does bind to RI (23) (60).
RNase A is responsible for many firsts in the modern understanding of proteins. The discovery of the effects of boiled pancreas extract on nucleic acids from yeast, in 1920, may have been the first time a protein was shown to serve as an enzyme (a catalyst to a chemical reaction) (61). In the late 1940’s, Armour, Inc., a hot dog company, was able to purify RNase A by the kilogram, a feat made possible in part due to RNase A’s exceptional resistance to high heat and acidity (55) (62). Armour distributed 10mg samples, for free, to any scientist who requested one. By the early 1960s, this wide availability led to several breakthroughs in the fledgling field of biochemistry. The catalytic mechanism of RNase A was the first enzymatic mechanism to be solved to the level of its amino acids (55). The amino acid composition of RNase A was also discovered before any other enzyme (17).

In 1952, a decade prior to any of these insights into the structure and mechanism of ribonucleases, Pirotte and Desreux (63) noted that the soluble fraction obtained from homogenized guinea pig livers was able to inhibit ribonucleolytic activity. By the late 1970s, this activity was found to be the action of ribonuclease inhibitor, which binds to and inhibits ribonucleases in a 1:1 stoichiometric ratio (64).

The affinity of an enzyme for its inhibitor, or how tightly they bind together, can be quantitatively represented by a value known as an inhibition constant, or $K_i$ (65). This value, measured in molarity, represents the concentration of inhibitor required to reduce the activity of its target by 50%; lower inhibition constants imply a higher affinity between two proteins. Lee and coworkers (66) used several techniques to detect the remarkably low $K_i$ for the RI·ribonuclease interactions and the method by which they interact. First, RI and a target ribonuclease, angiogenin (ANG, or human RNase 5), were incubated together in a 1½:1 ratio. A large excess of RNase A was added, which
served as a scavenger for free RI. Aliquots were removed at regular intervals to track the rate at which angiogenin became free in solution. This was repeated, with ANG and RNase A switching roles. The $K_i$ for the RI-ANG complex was found to be $7.1 \times 10^{-16}$ M, and the $K_i$ for the RI-RNase A complex was found to be $4.4 \times 10^{-14}$ M. This indicates that RI binds to ANG tighter than to RNase A (66). The addition of cytidine 2’-phosphate, a competitive inhibitor of RNase A which binds to its active site, decreased the association rate between RI and RNase A, suggestive of a competitive mode of binding (binding blocks the target’s active site) between RI and RNase A (66). Modifying the active site of angiogenin resulted in a weakening of the RI-ANG complex, also suggesting a competitive binding between RI and ANG (67).

RI binds ribonucleases tightly, but also quickly (68). This impressively fast rate is aided by Coulombic attraction, or the attraction between positive and negative charges. At biological pH, ribonucleases are cationic proteins (are positively charged), and RI is an anionic protein (69) (70). This leads to RI and its target ribonucleases being brought together faster than by diffusion alone (71).

In recent years, the determination of the 3D structure of the binding interaction between RI and angiogenin, eosinophil-derived neurotoxin, RNase A, and RNase has been determined by x-ray crystallography. Being able to “see” RI binding to its ligands has provided new insights into what makes RI such an exceptional scavenger of ribonucleases. From the data provided by these crystal structures, the exact location of hydrogen bonds and electrostatic interactions that participate in the binding interaction can be determined (Table 2).
Although RI is a large and repetitive protein, most of the binding interactions between RI and ribonucleases are concentrated very close to the C-terminus of RI (72) (74). The ribonuclease residues that participate in the binding interaction are regionally focused at the catalytic core rather than at any one end (70) (74). This regional focusing is the general rule, but the specific contacts being made between the residues of RI and a given ribonuclease are mostly unique. ANG and RNase A both make extensive contacts with RI, but only hold in common about 1/3 of those interactions (74). Additionally, there is no clear relationship between homology to RNase 1 (which human RI binds the strongest) and tighter binding (Table 2), which indicates that the ability of RI to inhibit ribonucleases is based on its ability to recognize unique characteristics of its targets rather than any structural similarities shared by the vertebrate-specific family.

<table>
<thead>
<tr>
<th>Ribonuclease</th>
<th>% Identity with RNase 1*</th>
<th>Kᵢ with RI (fM)</th>
<th># Contacts from RI</th>
<th># Contacts from RNase</th>
<th>Surface Area Buried by RI (Å²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNase 1</td>
<td>100</td>
<td>0.29</td>
<td>28</td>
<td>23</td>
<td>2802</td>
</tr>
<tr>
<td>RNase 2 (EDN)</td>
<td>28.4</td>
<td>2.7</td>
<td>42</td>
<td>31</td>
<td>3438</td>
</tr>
<tr>
<td>RNase 3 (ECP)</td>
<td>27.9</td>
<td>0.7</td>
<td>30</td>
<td>28</td>
<td>2659</td>
</tr>
<tr>
<td>RNase 4</td>
<td>40.6</td>
<td>4.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>RNase 5 (ANG)</td>
<td>33.3</td>
<td>N.D.</td>
<td>31</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>RNase 6</td>
<td>30.9</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>RNase 7</td>
<td>31.6</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>RNase 8</td>
<td>32.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>RNase A</td>
<td>68.0</td>
<td>44</td>
<td>28</td>
<td>24</td>
<td>2550</td>
</tr>
<tr>
<td>BS-RNase</td>
<td>70.3</td>
<td>&gt;2x10⁹</td>
<td>No Binding</td>
<td>No Binding</td>
<td>No Binding</td>
</tr>
</tbody>
</table>

Table 2: The affinity of RI for various ribonucleases. "N.D." indicates that a given value has not yet been determined [and/or published]. Lower Kᵢ values indicate tighter binding between RI and a given ribonuclease. Table is adapted from Rutkoski et al. 2008 (23) with additional data from Kobe and Deisenhofer 1996 (72).

* % Identity is the percentage of identical amino acid residues at identical positions when two proteins are compared with each other.
◊ No published Kᵢ exists for the interaction between RI and these ribonucleases. However, ECP has been shown to be “sensitive” to RI (73), RNase 6 binds to RI in a “very tight” interaction (74), and RNase 7 is also “sensitive” to RI. (75).
The size and shape of RI also play an important role in ribonuclease binding. The interface between RI and RNase 1 buries away 2,802 Å² (1 Å = 0.1 nanometers) of the surface area of the pair of proteins, including the catalytic core of the ribonuclease (23). This area is 100 billion times smaller than the period at the end of this sentence, but for a protein-protein interaction, this is pretty impressive. It’s almost twice the surface area buried between monoclonal antibodies that bind to proteins (a gold standard for tight binding) or between barnase and barstar, a bacterial ribonuclease/ribonuclease inhibitor pair (76). The non-globular horseshoe shape of RI allows the exposure of 10% more of its surface area than would be expected this protein, given its mass, allowing for a maximized binding surface (72).

One might imagine RI acts like a gigantic claw, trapping the ribonuclease inside of it and gripping it from all sides. RI does open wider to accommodate its target, but much of the ribonuclease binds instead to the top of RI, and some even manages to escape the “bite” of RI completely (77) (Fig. 3). RI does not have a single hinge point; rather, its entire structure stretches to accommodate ribonucleases (72).

The parallel β-strands on the interior of RI, which are unusually flat, are a hotspot for protein binding. They contribute 32% of the residues that bind RNase A, but only make up 11% of the total protein (77). β-strands may also be responsible for another protein-protein interaction in RI: dimerization, or the creation of a bound “pair” of RI-ribonuclease complexes. The N-termini of two RI molecules appear to flip upside-down to form β-strands which allow the two proteins to stack together (Figure 3). This
Figure 3: Binding Interactions with RI.

The top row of images shows a single RI-RNase pair from the top view. RI is shown in green. The bottom row of images shows a frontal view, including a dimeric RI-RNase pair with RI in gray. The RIaA complex exists as a monomer in free solution (103). Note the RI-RNase 1 complex, where this dimerization is most clear. Images created using PyMOL. Crystal structure for RI-RNase 1 from PDB #1Z7X (70). Crystal structure for RI-RNase A from PDB #1DFJ (72). Crystal structure for RI-RNase F from PDB #2BEX (178).

Crystal structure for RI-EDN from PDB #14AY (74). Crystal structure for RI-Angiogenin from PDB #14AY (74). Crystal structure for RI-RNase 1 from PDB #1Z7X (70). Crystal structure for RI-RNase A from PDB #1DFJ (72). Crystal structure for RI-RNase F from PDB #2BEX (178).
dimerization buries a significant amount of additional surface area \((1,700 \, \text{Å}^2)\) in the case of RI-RNase 1 \((70)\). However, it is not known if this dimerization actually occurs \textit{in vivo} or if it is simply a consequence of the crystallization process that is used to acquire the 3-dimensional structure of proteins.

\textit{Evolution of ribonuclease inhibitor, a leucine-rich repeat protein}

Ribonuclease inhibitor belongs to a special class of proteins which contain “leucine-rich repeats”, or LRRs, in their amino acid sequence. A leucine-rich repeat is a short amino acid sequence motif that is unusually rich in the amino acid leucine \((78)\). LRRs are usually 20 to 30 residues long, and in RI, they alternate between 28 and 29 residues \((78)\). The 28-residue LRRs in RI are named “A-type” repeats; the 29-residue LRRs are named “B-type” repeats \((21)\). LRRs are always found repeating in tandem, as few as 2 times and as many as 52 times in a row \((78)\) \((79)\) \((80)\).

The LRR sequence motif has been found in over 60,000 proteins from fungi, plants, animals, bacteria, archaea, and even viruses \((81)\). The LRR motif was first recognized in a protein called leucine-rich \(\alpha_2\)-glycoprotein, which was isolated from human blood serum \((79)\) \((82)\). Ironically, the precise function of this protein still has yet to be discovered; however, other LRR-containing proteins have since been found with have a vast array of roles including, but certainly not limited to, neuronal development, mRNA transport, cell signaling, cell adhesion, and apoptosis \((83)\) \((80)\). In these various roles, the LRR motif typically contributes to molecular recognition and protein-protein binding interactions, with the RI-ribonuclease interaction being a prototypical example \((84)\). In plants and animals, a class of LRR protein receptors has evolved that recognize and bind molecular features common to bacterial and fungal pathogens,
allowing a defense response; fungi may have evolved a similar yet unique defensive system that also relies on LRRs (85). In humans, the correct functioning of LRR-containing proteins is vital; mutations in over 30 proteins with the LRR motif have been attributed to diseases including Crohn’s disease, multiple sclerosis, arthritis, and susceptibility to Legionnaires’ disease (80).

The LRR motif is characterized by a highly conserved segment (HCS) followed by a variable segment. The HCS typically consists of either the sequence LxxLxLxxNxL or LxxLxLxxCxxL, where L is leucine, N is asparagine, C is cysteine, and x is any other amino acid (80). Occasionally, leucine is substituted by other hydrophobic residues including isoleucine, valine, or phenylalanine; similarly, asparagine and cysteine can be occasionally substituted by other polar, uncharged residues including serine, threonine, and also each other (81). Eight general classes of LRRs have been recognized based on shared patterns in variable segment (81). These eight classes are “RI-like”, “SDS22-like”, “Cysteine-containing”, “Bacterial”, “Typical”, “Plant-specific”, “TpLRR” and “IRREKO” (79). The “IRREKO” class (Japanese for “nested”) was the most recent to be documented and is closely related to the “SDS22-like” and “Bacterial” classes (81).

With the discovery of the 3D crystal structure of porcine RI in 1993, the structural role of the LRR motif was brought to light (21). Since then, the 3D structures of many other LRR proteins have been found. Although the LRRs in these proteins are diverse, the proteins themselves share very similar structural characteristics (Figure 4).
Figure 4: The 3D Structures of four other proteins that contain Leucine-Rich Repeats (LRRs), viewed from two angles. Helical structures are shown in blue; β-strands are shown as white arrows. Note the similarity to RI (Figure 2). Images were created using PyMol.

A) Single monomer of the protein decorin, which forms a dimer in vivo, from Bos taurus (cow). It is found as a member of the extracellular matrix and earned its name because it binds to and "decorates" collagen fibrils, which are vital to the integrity of connective tissue (224). From PDB#1XKU.

B) Internalin, from the bacteria Listeria monocytogenes, a food-borne pathogen that causes listeriosis. L. monocytogenes uses internalin to bind to e-cadherin, a cell surface receptor protein, in the intestines; once attached, the bacterial cell then induces its own phagocytosis into the intestinal cell (225). From PDB#1O6S.

C) Toll-like receptor 3, a member of the toll-like receptor (TLR) family, from Homo sapiens. These proteins function as a part of the innate immune system, recognizing and binding typical pathogen-related molecules, such as flagellin, a protein from bacterial flagella, or lipopolysaccharide (LPS), a molecule associated with bacterial cell membranes. Once such a trigger has been bound, TLRs activate an immune response. Toll-like receptor 3 binds double-stranded RNA, a molecule that is a sign of a viral infection (226). From PDB#2A0Z.

D) Single monomer of the protein YopM from the bacterium Yersinia pestis, which is responsible for the bubonic plague. In vivo, YopM forms a tetramer which Y. pestis injects into host cells, where it binds to and interferes with the action of host immune response proteins (227). From PDB#1JL5.
Like RI, their shape is curved, with each modular LRR unit contributing a \( \beta \)-strand to the internal \( \beta \)-sheet on the interior of the curve. On the external surface of the curve, each unit typically contains a helical structure (79). In RI, this helical structure is an \( \alpha \)-helix.

The LRRs in RI, at 28 or 29 amino acids in length, are uncommonly long, and the exterior portion of more typical LRRs, from 20 to 26 amino acids in length, are too short to form an \( \alpha \)-helix. Instead, these shorter LRRs usually contain other less-bulky secondary structures such as polyproline II helices, \( 3_{10} \) helices, \( \beta \)-strands, and \( \beta \)-turns; occasionally, short versions of these structures appear in tandem (Figure 5) (86). This reduced bulk allows other LRRs to relax into their banana-like shape, unlike the more tightly-wound RI (Figure 4).

![Figure 5: The various secondary structures of leucine-rich repeats.](image)

Note the variety of structures found on the external side of each repeat. -helices are in green, \( 3_{10} \) helices are in red, polyproline II helices are in blue, and \( \beta \)-sheets are in yellow. A) A 28-residue LRR from RI. B) A 20-residue LRR from YopM. C) A 21-residue LRR from decorin. D) A 22-reisude LRR from InlH. E) A 23-residue LRR also from decorin. F) A 24-residue LRR from Toll-like receptor 3. Figure adapted and colorized from Bella et al. (86).
Given that LRR proteins appear throughout the spectrum of biological life, and even in viruses, it has been argued that all LRR proteins share a common evolutionary ancestor, descending from one gene that would have existed billions of years ago, before the branching of lifeforms into separate domains. Due to the observation that the known LRRs can be sorted into eight tightly-conserved classes that appear in specific taxa, Kajava (87) asserts that each class of LRRs arose independent of one another during the course of evolutionary history. Additionally, he points out that the hydrogen bonding network between LRRs within a protein, which lends to their structural stability, cannot be formed between LRRs from separate subgroups. However, using a novel grouping method based on sequence homology, Andrade and coworkers (88) was not able to divide the various LRR motifs into separate classes as cleanly as Kajava. Matsushima and coworkers (89) discovered LRR proteins that contain “supermotifs” of several LRRs from different classes repeating in tandem. These supermotifs contain LRRs from the typical and bacterial subgroups, but were paradoxically found in proteins from eukaryotes (89). In addition, the IRREKO class of LRRs appears to have evolved from a common ancestor also shared by the bacterial and SDS22-like LRR subgroups (81). Combined, these findings tie together the SDS22-like, bacterial, typical, and IRREKO LRR classes into one evolutionary family that may contain some human LRR proteins. This provides support for the theory that LRRs have a common ancestor, but exactly where RI, which defines its own class of eponymous LRRs, fits in this eons-old story is not well understood.
The modular nature of RI also provides clues to its more recent evolutionary history. The gene for human RI has evolved through the duplication of exons, the protein-coding segments of our genes. In eukaryotes, genes are made of segments called exons and introns; the introns are cut out and the exons spliced together to make the final mRNA transcript which is, in turn, translated into a protein (Figure 6). In the gene that codes for RI, each internal exon (i.e., excluding the ones at either end) codes for exactly 2 LRRs: one A-type and one B-type repeat, a total of 57 amino acids (90). All of the introns are also in the same phase, meaning that if any of the internal exons are removed or moved around, the LRRs in the remaining exons would still remain aligned with each other (90). In fact, mutated forms of RI have been expressed with various internal exons removed; remarkably, these mutants retain inhibitory activity (91).

In addition to their identical length, the internal exons are also homologous with each other, with 50-60% sequence identity. These observations cement the support for the exon duplication theory. Haigis and coworkers (90) used various methods of phylogenetic analysis to show that the internal exons of RI are also extremely stable, and have not homogenized over time to become so similar to one another. This implies that the exon duplication event happened very early in the evolutionary history of RI and the internal exons have remained similar over time.

**Figure 6: The exon-intron structure of eukaryotic genes.** UTR stands for "untranslated region"; this part of the mRNA transcript merely flanks the gene and is not part of the code for that mRNA's corresponding protein. Image created by Wikipedia user Qef (235) and uploaded in the public domain.
Another perspective on the evolutionary history of RI can be garnered by studying the evolutionary history of its target proteins, the vertebrate-specific ribonucleases. It was originally thought that these proteins were only present in tetrapods (four-limbed: amphibians, reptiles, birds, and mammals); however, more recent genetic analyses have allowed for the identification of such ribonucleases in bony fish, but not cartilaginous fish (92). This divide is thought to be the first major taxonomic divide in the history of vertebrates, occurring approximately 420 million years ago, during the Devonian period (93). It may be around this time that the first RNase A-like proteins arose (Figure 7). Thus, except for cartilaginous fish, vertebrate-specific ribonucleases appear throughout the subphylum Vertebrata; together, this group of

![Figure 7: Model of the evolution of modern vertebrates.](image)

Note that sharks and other related cartilaginous fish are the only clade not to express RNase A homologs, hence the likely concurrent evolution of RNase A homologs and a bony skeleton. Points in time where RI could have arisen (Either alongside the RNase A family or during the rise of mammals, the only clade from which RI has yet to be isolated) have been highlighted. Image adapted from *Understanding Evolution* (234) with permission from the University of California, Berkeley.
animals are termed *teleostomes*. Although these ribonucleases are highly divergent, enough of their structure has been conserved since this ancient taxonomic split that human RI is able to bind to vertebrate-specific ribonucleases found in zebrafish (94). Humans and zebrafish, both teleostomes, have experienced hundreds of millions of years of genetic divergence, and yet this RI-ribonuclease interaction has been remarkably conserved. Oddly enough, human RI does not inhibit vertebrate-specific ribonucleases isolated from frogs or chicken, suggesting that these ribonucleases have diverged in some classes more intensely than in others (90).

Given that vertebrate-specific ribonucleases, in the absence of RI to prevent their activity, are cytotoxic (25), it appears vital for a protein like RI to arise and evolve alongside these ribonucleases. Yet, the mechanism that various teleostomes use to inhibit their vertebrate-specific ribonucleases remains to be determined. Other organisms, including bacteria and protozoans, express both intracellular and extracellular ribonucleases unrelated to vertebrate-specific ribonucleases; these species also express inhibitor proteins for these ribonucleases that are equally unrelated to human RI (95) (96). Hence, the RI/ribonuclease coupling is not a unique schema but rather a sign that the evolution of an inhibitor to regulate RNA-cleaving proteins is typical and important. Using the gene for human RI as a search template in the NCBI biosystems database, it appears that homologs of RI may be present in as distant of species as *Gallus gallus* (chicken), *Taeniopygia guttata* (Zebra finch), and *Anolis carolinensis* (Carolina anole, a small lizard) (97) (98) (99). Yet, to date, homologs to human RI have only been physically isolated from mammals. Thus, it is not known if the proteins responsible for inhibitory activity in other teleostomes have any structural similarity to RI (18). Complicating things further is the fact that the ancestry of RI is tied
to its ancestry as an LRR protein. This places it on a historical timeline that may have begun long before the rise of eukaryotes, let alone animals or the vertebrate-specific ribonuclease family.

**Cysteine-mediated oxidation sensitivity**

The efficacy of RI to bind to and arrest the activity of members of the vertebrate-specific ribonuclease family is remarkable, but RI has one weakness: oxygen, or more specifically, reactive oxygen species (ROS) such as hydroxyl radicals or hydrogen peroxide (100) (101). This weakness is derived from its high cysteine content. Through an oxidation reaction, the thiol groups from two cysteines form a new covalent bond known as a disulfide bridge (Figure 8). Disulfide bridges serve as structural linkers and are typically found in secreted proteins, where they aid in maintaining structural integrity in the exotic and dangerous extracellular world. However, in RI, the oxidative formation of these disulfide bonds leads to a structural collapse and a complete loss of inhibitory function (24). Human RI contains 32 free cysteines, all of which are free and reduced in the native, active form of RI (102). This number of cysteines, for a cytosolic protein, is unusually high (24).
vitrō analysis has shown that the oxidative collapse of RI is cooperative and rapid (24). The oxidation of only a handful of cysteines leads to the formation of an intermediate structure, due to the new disulfide bonds. This change in conformation increases the reactivity of the free cysteines that remain, leading to the full pairing of all of the cysteines in RI (24).

There is truly something ironic about the oxidation sensitivity of RI. RNase A owes its early discovery and long, extensive history of research to its remarkable stability to a wide variety of environments and reagents; had it not been for the important relationship between RI and RNase A, it may have not been discovered nearly as early or researched quite as comprehensively, thanks to its contrasting fragility.

**Biological Roles of Ribonuclease Inhibitor**

*Distribution within the body and within the cell*

RI appears to be ubiquitous throughout the body. In mammalian studies, RI has been found within the cells of many organs, including the kidneys, thymus, esophagus, testes, placenta, brain, lung, spleen, heart, adipose tissue, skin, and red blood cells (49) (103) (104) (105) (106) (107) (108) (109) (110) (111). However, RI is not found in extracellular fluid (108).

The localization of RI within the cell has only recently been revealed. An early study by Chakravorty and Busch (112) in 1968 found that RI could be found both in the cytoplasm and in the nucleus. However, in 1972, Roth and Juster (113) failed to find RI in the nucleus by several methods. Therefore, it had been widely accepted that RI is found exclusively in the cytoplasm (22) (70). However, the use of modern visualization
techniques such as confocal microscopy has proved that Chakravorty and Bush were right all along. In 2011, it was discovered that RI is not localized to the cytoplasm, but to the nucleus and mitochondria as well (114). This opens up the possibility that unexplored roles for RI exist throughout the cell.

*Role in cell protection against ribonucleases*

The cytotoxicity of vertebrate-specific ribonucleases was first discovered over 50 years ago, when it was shown that RNase A could stop the growth of malignant cells both *in vivo* and *in vitro* (115). The therapeutic potential of this discovery was limited, however, because the required dosages were far too large. Since then, other vertebrate-specific ribonucleases have been discovered that possess more potent cytotoxic effects. One of these is bovine seminal RNase (BS-RNase). BS-RNase has an 81% sequence identity with RNase A, but it holds a unique place within the vertebrate-specific ribonuclease family (116). Unlike any other known member of this family, BS-RNase forms a dimer *in vivo* which exists in equilibrium between two isomers, M=M and MxM; in the latter, the N-termini of each monomer are interchanged (117)(Figure 9). BS-RNase has significant anti-tumor activity, which the MxM isomer is responsible for (117) (118) (119). However, the toxic effects of BS-RNase are not completely specific. BS-RNase suppresses the immune system, is toxic to embryos, and causes male sterility (120) (121) (122) (123). These negative attributes may negate the possible medical uses of this protein.
Another vertebrate-specific ribonuclease with cytotoxic effects is Onconase®, also known as ranpirnase, P-30, or simply ONC. This protein is currently in development as a candidate anti-cancer and antiviral drug (124). ONC is found in the liver of *Rana pipiens* (common name: northern leopard frog) (125) (126). As mentioned earlier, vertebrate-specific ribonucleases are cationic (positively charged); ONC’s anti-tumor selectivity is postulated to be a result of the increased anionic (negatively charged) nature of the surface of cancerous cells (127). This greater difference in charge allows for increased translocation of ONC across the cell membrane and is critical to ONC’s cytotoxicity (128) (71).

Figure 9: 3D Crystal Structure of the MxM isomer of dimeric BS-RNase. Monomers are orange and cyan, respectively. The helices intertwined into the other monomer’s tertiary structure represent the interchanged N-termini of each monomer. Image created using PyMol from PDB#1BSR (228).
The cytotoxicity of BS-RNase and ONC both appear to be mediated by their ability to evade RI (129). RI can bind to monomers of BS-RNase, which are not cytotoxic; however, RI cannot bind to the MxM dimer, which is cytotoxic (130). Furthermore, variants of BS-RNase which cannot dimerize or be bound by RI are even more cytotoxic than the wild-type dimer, which suggests that not only is avoiding RI an important factor in cytotoxicity, but also that the wild-type dimer’s cytotoxicity is dampened by its occasional dissociation into RI-inhibited monomers (131). ONC lacks several residues that RNase A and its human counterparts contain, residues which appear to be important to RI binding; its low sequence identity (only 28%) is postulated to be responsible for its ability to evade RI (132) (133). G88R RNase A (an RNase A
variant in which the residue 88, glycine, has been replaced with arginine) displays both reduced affinity for RI and increased cytotoxicity when compared to wild-type RNase A (129). Increasing the amount of RI expressed in K562 and HeLa cells (cultured human cancer cell strains) decreased their sensitivity to G88R RNase A. The silencing of RI through RNA interference had the inverse effect (25). The relationship between resistance to inhibition and cytotoxicity has earned RI the title of “intracellular sentry”, with a proposed biological role of guarding cells from rogue ribonucleases (129). Without its presence, endogenous ribonucleases that manage to enter the cell could otherwise be cytotoxic, as appears to be the case when RI-resistant ONC or BS-RNase are endocytosed (Figure 10).

Whether RI is truly an “intracellular sentry” has been controversial. If RI is the failsafe that prevents ribonucleases from degrading important cellular RNA, then silencing RI should make increase the cytotoxicity of ribonucleases. Monti and D’Alessio silenced the expression of RI in HeLa cells to undetectable levels; this further increased the vulnerability of these cells to BS-RNase (134). Conversely, this did not have any effect on the cytotoxicity of RNase A. It was concluded that evasion of RI may not be the mitigating factor in whether or not a ribonuclease can cause harm, and that only certain vertebrate-specific ribonucleases are intrinsically cytotoxic. However, it has been suggested that, even at undetectable levels of RI, the RI-RNase A inhibition interaction is so strong that the cytotoxicity of RNase A can still be prevented (25). Conflictingly, engineered dimers of RNase A show cytotoxic effects yet were tightly bound by RI in vitro, which completely inhibited their RNA-cleaving activity (135). However, such an RNase A dimer has yet to be found in nature (92). Variants of vertebrate-specific ribonucleases which were engineered in a way that may mimic
naturally-occurring post-translational modifications show reduced binding to RI concurrent with increased cytotoxicity (23). Taken as a whole, the available evidence suggests that the activity of vertebrate-specific ribonucleases can be potentially toxic, hence the need for an “intracellular sentry” like RI.

**Role in managing oxidative stress**

Although the inside of a cell is usually a reducing environment, an increase in the concentration of ROS can lead to a state of oxidative stress (136). ROS are naturally produced as a result of ATP production in the mitochondria but can also be generated in excess by the presence of compounds that lead to the production of excess ROS or by certain disorders (137) (138). Such oxidative stress can also lead to the breakdown of RI in vivo. Treatment of LCC-PK1 cells (from pig kidney) with hydrogen peroxide and/or diamide, an oxidizing agent that specifically targets thiol groups, found a significant decrease in the activity of endogenous RI (136). The concentration of RI in cells suffering oxidative stress also decreased significantly faster than in control cells, independent of mRNA transcript levels; this suggests that oxidized, deactivated RI is specifically targeted for proteolysis. Whether this process is regulated by the ubiquitination (molecular tagging) of denatured RI or the specific, direct targeting of oxidized proteins by the proteosome is still unknown (139).

Glutathione, a 3-residue polypeptide, is the cell’s chief mechanism to maintain a reducing environment in the cytoplasm. Free glutathione can be oxidized by ROS within the cell and reduced back to its original state by glutathione reductase (140). Reduced glutathione is simply a reduced cysteine with a flanking amino acid on either side, a sign of the antioxidant efficacy of the cysteine residue. As discussed earlier,
active human RI contains 32 reduced cysteines. Thus, it is possible that RI plays an important role in regulating the redox state of the cell, especially considering its ubiquitous nature. Wang and Li (141) showed that RI sourced from cow placenta had strong antioxidant effects against several ROS, including superoxide anions, hydroxyl radicals, singlet oxygen, and lipid radicals. The scavenging activity of RI against these ROS was stronger than that of tea polyphenols, the compounds responsible for green tea’s supposed antioxidant health benefits (142). These antioxidant effects have also been found in vivo. When RI was overexpressed in rat glial cells, a significant increase in cell viability in the face of oxidative injury (caused by hydrogen peroxide) was reported (143). Mice given injections of RI over the course of 5 days were also better protected against oxidative liver damage induced by carbon tetrachloride. HUVE (cultured human endothelial cells) and HeLa cells deprived of RI through RNA silencing also show a decreased amount of intracellular GSH and an increased amount of oxidation-induced DNA damage following induced oxidative stress (144). The recent discovery of the presence of RI within the matrix of the mitochondria, where much of a cell’s ROS are produced, lends further credence to the antioxidative role of RI (114).

RI also appears to be directly involved in oxidative stress signaling pathways. Cytoplasmic RI in mouse embryonic fibroblasts is rapidly degraded during oxidative stress, concurrent with a significant reduction in overall protein synthesis (independent of eIF2α phosphorylation, a known stress-induced mechanism which reduces protein synthesis) and an increase in the site-specific fragmentation of transfer RNAs (tRNAs) (145). This tRNA fragmentation is caused by angiogenin, which cleaves tRNAs within their anticodon loops to produce specific small RNA molecules known as tiRNAs (146). tiRNAs inhibit protein synthesis by displacing the eIF4F complex (responsible for
bringing mRNA to the ribosomes for translation) from mRNA; injection of synthetic tiRNAs was sufficient to inhibit protein synthesis by 20% (145) (147) (148). Silencing the expression of RI leads to the production of tiRNAs even in the absence of induced oxidative stress, while overexpressing RI prevents the production of tiRNAs (46) (146). It appears that the oxygen sensitivity of RI serves as a cell’s signal that it is under oxidative stress; once RI has been oxidized and degraded, angiogenin is then free to create tiRNAs and reduce the rate of protein synthesis, which may increase the likelihood of cell survival (149). This newly-discovered signaling pathway has yet to be fully understood.

Although free RI oxidizes and denatures in a complete, all-or-none fashion, the same is not true of RI while it is complexed with ribonucleases. When porcine RI bound to RNase A was treated with DTNB, an oxidizing agent, RI was found with 14 of its 30 cysteines oxidized; such an intermediate state cannot be found for free RI (150). In this partially-oxidized complex, RNase A was only inhibited by 85%. Johnson and coworkers (151) further confirmed this phenomenon by finding a large increase in both thermal and oxidative stability when either human or bovine RI were bound to RNase A (bovine) or RNase 1 (human). If RI plays dual roles, both protecting the cell from rampant ribonucleases and protecting the cell from oxidative stress, it would then appear that the former takes precedent. Eukaryotic cells have a vast array of enzymes and small molecules at their disposal to protect themselves against oxidative stress, with RI being only one of them (152). On the other hand, RI is the only shield available against possibly-cytotoxic ribonucleases (151). Thus, the increased resistance to oxidation when holding on to a ribonuclease is likely a trait selected for to make sure RI keeps its priorities straight.
Ribonuclease inhibitor and cancer

Whether or not RI serves to aid in the proliferation or prevention of cancer is still under investigation. Protecting the cell from oxidative damage is sufficient reason on its face to see RI as an anti-cancer protein; oxidative damage is closely linked to the development of cancer (153). Yet, once cancer has developed, RI may actually be harmful, protecting malignant cells from oxidative damage that could otherwise slow their growth. Nobel laureate James Watson recently put his weight behind a new hypothesis that our cultural obsession with anti-oxidants may be harming cancer patients, as the effectiveness of many cancer treatments relies on their ability to induce the production of damaging ROS (154). This idea could place endogenous antioxidants, such as RI, in a new light. Resistance to histone deacetylase inhibitors (HDACis), a class of drugs which rely on ROS production to slow cancer development, has been attributed to an upregulation of RI expression (155). Still, RI is more than just an antioxidant, and has a place in other cellular mechanisms which affect the development of cancer in opposing ways.

One of these mechanisms is the regulation of angiogenin. Angiogenin may play a role in the oxidative stress response, but it was originally named for its role in angiogenesis, or the development of blood vessels. Angiogenin was first purified from human carcinoma cells in 1985 and was shown to be sufficient to initiate blood vessel growth (156)(Figure 11). Angiogenin’s RNA-cleaving activity, although weaker than that of RNase A or RNase 1, is necessary for angiogenesis (157). Equally necessary for angiogenesis is the translocation of angiogenin to the nucleus, where it serves as a transcription factor, stimulating the transcription of ribosomal RNA (rRNA) (44) (158), which is perhaps an ironic function for a ribonuclease. While normal levels of
angiogenin are important for proper cell growth and maintenance, abnormally high levels of angiogenin are found in cancer cells, where it serves at least two functions: increasing the level of rRNA to meet the increased demand for ribosomes to synthesize protein, and initiating blood vessel development to increase the supply of nutrients to the growing tumor (44) (159) (160) (161) (162) (163). RI, as a strong inhibitor of angiogenin, regulates the neovascularization that angiogenin induces (164). RI has also been shown to slow the growth of tumors, which has been attributed to this anti-angiogenesis function (165). The presence of RI in the nucleus may also signify that it is able to police the production of rRNA that angiogenin upregulates (114). RI is vital in maintaining the careful balance of angiogenin’s proliferative effects, thereby preventing cancerous cell growth.

New evidence suggests that RI is involved in another cancer-related pathway that, as of yet, is apparently unrelated to its role either as an inhibitor of ribonucleases or its antioxidant qualities. Kim and coworkers (166) discovered that cytosolic RI binds to PTEN, a phosphatase protein with a multitude of anti-tumor functions (167). Evidence also suggests that RI interacts with the Drosha complex, a protein complex found in the nucleus which is involved in the processing and maturation of microRNAs (166). The presence of nuclear RI increases the processing of miR-21, a microRNA which regulates many tumor suppressor genes, including PTEN; aberrant miR-21
expression and processing is involved in tumor growth (168). Together, it appears that PTEN binds RI and prevents it from localizing to the nucleus and associating with Drosha, thereby decreasing the expression of miR-21. More research is needed to discover the exact mechanism and relationship between Drosha, PTEN, and RI.

RI has a complicated role in the regulation of malignant cell growth. It has been found to be downregulated in breast cancer cells, and the silencing of RI expression in bladder cancer cells increased their growth and potential to metastasize (169) (170). Additionally, the upregulation of RI expression increased the rate of apoptosis in breast cancer cells and decreased the invasive potential of melanoma cells (171) (172). Nevertheless, its ability to block the action of various cancer treatments and its role in promoting miR-21 processing provides evidence that RI may actually aid in cancer proliferation.

While research concerning the direct role of RI in cancer development remains contradictory, the understanding of RI-ribonuclease interactions has still furthered the development new anti-cancer drugs. QBI-139, a RNase 1 variant currently in Phase 1 human trials, was specifically designed to be cytotoxic by evading RI (173) (174).

Other biological roles

The number of important functions attributed to vertebrate-specific ribonucleases continues to rise. As it does, the possible roles RI may play in vivo increases in tandem. Many of these roles involve angiogenin, a versatile ribonuclease. For example, RI is overexpressed in the placenta of women with pre-eclampsia; this may be a sign that angiogenin is being improperly inhibited, causing the failure of correct blood vessel development that characterizes this disorder (175). Angiogenin also plays an
important and newly discovered role in the proper growth and maintenance of neurons, and mutations in the angiogenin gene have been reported in patients with amyotrophic lateral sclerosis (ALS), a neurodegenerative disease (41) (45) (176). This discovery may foretell a regulatory role for RI in neuronal development as well. RNase 4 has also been discovered to share many functions with angiogenin (RNase 5), including angiogenesis and the regulation of neuronal development and vitality, broadening the possible importance of RI in these regulating these tasks (39).

**Investigating RI by Constructing Oxidation-Resistant RI Variants**

The biological roles that have been discovered for RI revolve around two of its defining characteristics: its potent inhibition of vertebrate-specific ribonucleases, and its vulnerability to denaturation by ROS. These two characteristics may be fundamentally intertwined, arising from the same amino acids. On the contrary, it is also possible they arise from unrelated regions of the protein, making it imaginable that a modified version of RI could continue to bind ribonucleases while being immune to oxidation, or vice versa. Learning more about the relationship between these functions could provide us with hints about the evolutionary history of RI and the biological importance these functions hold within living organisms. In this study, we seek to learn more about the role of RI’s oxidation-sensitive Achilles’ heel: its cysteines.
Manipulating RI: where and how?

To learn more about the role of cysteines in human RI, modified versions of this protein have been designed in which sets of these cysteines have been replaced. Five repeating cysteine positions exist in RI: positions 10, 17, and 21 in the A-type repeat, and positions 21 and 29 in the B-type repeat (Figure 12) (22). In addition, RI contains 6 other cysteines that do not appear in any pattern. Six variants of RI were designed by Dickson and Krawicz in which substitutions were made for the cysteines that fit into each of these patterns (Table 3) (177).

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**Figure 12: The Various Patterns of Cysteine’s Appearance in the Secondary Structure of Human RI.** Here, the amino acid sequence of RI has been formatted to show the alignment of each LRR module. The cysteines in RI can be grouped into several clear patterns: A10 cysteines, in orange; A17 cysteines, in light green; A21 cysteines, in pink; B21 cysteines, in dark green; and B29 cysteines, in blue. Cysteines shown in red do not fit into any repeating pattern. Image from Krawisz (177).
<table>
<thead>
<tr>
<th>Variant Name</th>
<th>Cysteines Replaced</th>
<th>Replaced With</th>
<th>Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10</td>
<td>Cysteines at A10 Position</td>
<td>Serine</td>
<td>In β-turn, where it may be forming H-bond with backbone carbonyl (74). Serine replaces –SH with -OH, maintaining H bond. Serine also at this position in other LRRs (84).</td>
</tr>
<tr>
<td>A17</td>
<td>Cysteines at A17 position</td>
<td>Valine</td>
<td>Part of hydrophobic, non-solvent-exposed core; hydrophobic residues at this position in other LRRs (84). Conserves the bulk that the replaced sulfur atom provided.</td>
</tr>
<tr>
<td>A21/B21</td>
<td>Cysteines at A21 and B21 positions</td>
<td>Serine</td>
<td>Located in α-helices, is minimally solvent-exposed; therefore, a hydrophilic –OH containing residue was used (74).</td>
</tr>
<tr>
<td>B29</td>
<td>Cysteines at B29 position</td>
<td>Threonine</td>
<td>Located in β-turn, similar to A10 cysteines. Threonine was chosen instead of serine due to its appearance in this position in other LRRs (84).</td>
</tr>
<tr>
<td>Non-repeating</td>
<td>Cys11, Cys29, Cys74, Cys95, Cys328, Cys408</td>
<td>Various (See Legend)</td>
<td>Conservative substitutions made as described in text.</td>
</tr>
<tr>
<td>Cysteine-Free</td>
<td>All</td>
<td>As Shown Above</td>
<td>---</td>
</tr>
</tbody>
</table>

Table 3: The Design of Six RI Variants with Substitutions for Cysteine. Residue conservation from other LRRs based on sequences from Stumpp et al. (84). Structural information based on crystal structure of human RI bound to angiogenin (74).
The substitutions made at each site were carefully chosen in an attempt to maintain the native structure of RI. Structural data available from the crystallization of RI bound to angiogenin by Papageorgiou and coworkers (74) and an extensive analysis of amino acids conserved at these positions in other LRR proteins performed by Stumpp and coworkers (84) provide the basis for the decisions that were made in designing the variants. Cysteines at the A10 position are located in a β-turn and are solvent-exposed (in contact with the polar aqueous solution around the protein) and other LRRs contain polar residues here, including asparagine, threonine, and serine. For this reason, serine was chosen to replace the cysteines at the A10 position. Cysteines at the B29 position are also in a β-turn, but were instead substituted with threonine based on its appearance at this position in other LRRs. The cysteines at the A21 and B21 positions are in similar structural positions within α-helices, and were combined into one variant, in which they were substituted with a polar amino acid, serine, based on their solvent exposure. Cysteines at the A17 position were replaced with the hydrophobic amino acid valine based on this residue's conservation at this position in other LRRs and its position in the hydrophobic core of RI. The cysteines replaced in the “non-repeating” variant do not share similar structural roles. Cysteines 11, 29, 74, and 408 are solvent-exposed; nevertheless, they were replaced with alanine, in order to avoid increasing the polarity near the N-terminus and inducing any non-specific binding interactions. Cysteine 95 is in a similar position; it was replaced with a threonine to conserve bulk in this region. Cysteine 328 is within an α-helix and thus was also replaced with a threonine to conserve possible hydrogen bonding interactions. Cysteine-free RI contains all of the aforementioned substitutions.
While two cysteines in RI, Cys11 and Cys408, have been shown to be contact residues in the binding of various ribonucleases, it is possible that other cysteines may also be involved in more transient yet still significant interactions (72) (74) (178). Therefore, until each variant is fully characterized, it is not possible to know what effects each substitution for cysteine may have on either the structure or the function of RI. Noting that neither Cys11 and Cys408 are in one of the regular, conserved cysteine positions in their respective modules, one may hypothesize that the conserved cysteines in each module are important for the maintenance of secondary structures, while the non-repeating cysteines are free to form hydrogen bonds or van der Waals interactions with ribonucleases (19). It also may be true that the cysteines at the A10 and A17 positions are most vital for structural stability, given the relatively high frequency of cysteine conservation at this position throughout the modules of RI. The B29, B21, and A21 positions contain a cysteine residue at lower frequencies, in that order (19). However, at none of the aforementioned positions is cysteine present in every module of RI; thus, it may be possible to replace each cysteine at these positions, as we have done, and maintain the same protein structure. Once these variants have been expressed and purified, they will be characterized for their ribonucleolytic activity, their thermal and oxidative stability, and their conservation of structural components in comparison to wild-type RI.

**Optimizing Expression and Purification of Recombinant RI**

*Difficulty of RI purification*

The successful study of a protein *in vitro* requires two major steps: its production and its purification. This is equally true for RI, but it is not as easy as your everyday
protein. While RNase A is expressed in bovine sources in large quantities and can be purified from high heat and low pH conditions, RI is both less abundant and far more fragile. First, a source with adequate amounts of RI is required. In the past, RI has been purified from homogenized placenta; this is not an option when it comes to procuring variants of human RI (103). Presently, a recombinant expression system, in which the gene for RI is transformed into easily-grown cells such as common bacterial and yeast strains, is a much more popular choice. Second, the labile nature of the highly oxygen-sensitive structure of RI means that care must be taken to maintain reducing conditions and low oxygen concentrations at all times during the purification (179). Some expression protocols for RI have required 4 liters of recombinant bacterial culture simply to acquire a single milligram of purified RI, which has led some researchers to resort to attempting RI expression in eukaryotic insect cells (180) (181). In contrast, methods exist to purify over 100mg of RNase A from a single liter of culture (182).

In this study, much time has been devoted to optimizing an expression and purification system for human RI. A bacterial expression system in Escherichia coli has been chosen, due to the ease and affordability of culturing these cells in large quantities, the large number of E. coli strains that have been developed for protein expression, and the large knowledge bank that exists about optimal growing conditions for this model species. Other steps have been taken to ensure a maximum yield for this difficult, elusive protein.
**MBP as a solubility enhancer**

While the homogenous overall molecular structure of DNA makes the purification and processing of various genes simple and generalizable, the diverse chemical nature of the proteins they encode necessitate the development of a unique purification protocol for each new target. An indispensable tool in increasing the throughput and ease of protein purification has been the use of affinity tags. Affinity tags are auxiliary proteins or short polypeptides that are expressed together as a single product (a fusion protein) with a protein of interest; the affinity tag usually has some intrinsic quality that makes it easy to purify (183). This intrinsic quality sets a fusion protein apart from the thousands of other proteins that may be expressed within a cell. Maltose-binding protein (MBP) is a common affinity tag with an ability to bind to maltose or amylose, which can be attached to a resin capable of isolating MBP fusion proteins to 70-90% purity (184) (185) (Figure 13). MBP-fusions also tend to display impressive increases in solubility over wild-type target proteins (186) (187). Low solubility of expressed proteins in *E. coli* has been a major roadblock for

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**Figure 13: Expression and Purification of MBP Fusion Proteins.** A target gene, in this case human RI, is spliced into a commercially-available MBP vector plasmid (circular ring of DNA). In *E. coli*, this plasmid then expresses an MBP-RI fusion protein, which can be purified from cell lysate due to MBP’s binding to amylose. RI is then be cleaved off by the use of a specific protease, such as enterokinase. The target protein can be purified from a mixture with MBP by ion exchange chromatography. Image reprinted from (2013) www.neb.com from New England Biolabs (229). ©2013 New England Biolabs.
high expression and purification yields (188). This is especially true for RI, which is not only poorly soluble when expressed alone in *E. coli*, but also is not efficiently recovered from its insoluble form. Yields of over 30mg of soluble, active RI per liter of culture have been reported via the use of an MBP-fusion vector along with production at low temperatures (189). For this reason, a cleavable MBP fusion protein vector was chosen for the expression of human RI in this study.

*Chaperones as co-expression partners*

Another secret weapon that can increase the expression of active, soluble RI in *E. coli* is the co-expression of chaperone proteins, which aid in the proper folding of a target protein (190). Chaperones from *E. coli* named GroEL and GroES are commonly over-expressed to improve folding of recombinant proteins. *In vivo*, 14 monomers of GroEL form together in a large, double-ringed barrel-like structure, which is capped by a ring of 7 GroES monomers (191)(Figure 13). It is thought that this barrel structure encapsulates unfolded or misfolded proteins through hydrophobic interactions, providing a favorable environment for their folding; yet, the GroEL/ES complex mediates the folding of proteins that are too large to fit inside, leaving their complete mechanism of action unknown (192) (193). Co-expression of GroEL and GroES alongside wild-type RI has allowed for the production of up to 25 mg of active, folded protein per gram of dry cell weight (194). For this reason, GroEL/ES co-expression will be incorporated into the RI expression protocol in this study.
Manipulating redox conditions to optimize RI production

Finally, although cells are usually quite robust at internally maintaining a reducing state, the high oxidation sensitivity of RI can still lead to a significantly reduced yield in standard shake-flask cultures. This concern was addressed by Šiurkus and Neubauer, who were able to significantly improve the recovered amounts of active wild-type RI by spiking the culture media with DTT, a powerful reducing agent, and reducing the incubation temperature from the standard 37°C (body temperature) to 22°C (room temperature) (195). They theorize that the solubility of the expressed RI is increased not only by DTT’s antioxidant potential, but also by the slowed rate of expression.
caused by the combination of low temperatures and the toxicity of the high levels of DTT used. Because of the increased yields reported via this protocol, DTT-mediated regulation of the shake-flask cultures’ redox state will be used in this study, along with a reduced expression temperature.

**Characterizing RI and RI Variants: Activity, Structure, and Stability**

The goal of our RI variant design is to reduce the oxidative sensitivity of RI while maintaining activity and structure. While care was taken to make conservative substitutions for cysteine at each of its positions in RI, it’s not possible to know the exact effects of these substitutions on paper. Likewise, the impact each substitution may have on the oxidative stability of RI is not apparent. Therefore, once expressed, each variant will be evaluated for its maintenance of inhibitory activity, its resistance to oxidation-induced inactivation, its thermal stability, and its structural similarity to wild-type RI. This will allow us to evaluate the contribution to these characteristics made by the cysteines found at each conserved position.

_Determining the inhibitory activity and oxidative resistance of RI variants_

As discussed earlier, the activity of a competitive inhibitor against its enzyme target is represented by a value known as an inhibition constant, or $K_i$. This value represents the concentration of inhibitor which is able to reduce enzymatic activity by 50%. To find the $K_i$ of each variant, we will measure the activity of RNase A in its presence using a hypersensitive substrate whose cleavage can be detected fluorescently in real time (196). This fluorogenic substrate, 6-FAM-dArUdAdA-6-
TAMRA, contains a chain of nucleotides featuring the same cleavage site for RNase A: a ribonucleotide followed by a deoxyribonucleotide, 5’ to 3’ (Figure 15). The detection of this cleavage is possible due to a Förster resonance energy transfer, or FRET, interaction between the FAM (6-carboxyfluorscein) and TAMRA (6-carboxytetramethylrhodamine) fluorophores at either side of the molecule. The emission range of FAM overlaps with the excitation range of TAMRA, allowing the energy absorbed (as light) by the FAM to be transferred to TAMRA rather than released as light in FAM’s emission spectrum (Figure 16) (197). The nucleotide backbone of 6-

![Molecular structure of 6-FAM-dArUdAdA-6-TAMRA](image)

Figure 15: The molecular structure of 6-FAM-dArUdAdA-6-TAMRA, a sensitive substrate for detecting RNase activity. The nucleotide backbone allows for TAMRA to be held in close enough proximity to FAM to quench its fluorescence. The 3’ phosphate group on the ribonucleotide, Uridine, presents a bond scissile by members of the vertebrate-specific RNase family. Cleavage of this bond allows FAM to move freely from TAMRA, bringing an end to the quenching of its fluorescence. Modified from Kelemen et al. (196).
FAM-dArUdAdA-6-TAMRA holds FAM and TAMRA close enough together to allow a FRET interaction to occur; however, in the presence of an active ribonuclease, this backbone is severed, and the excitation of the freed FAM instead results in the release of light at FAM’s emission wavelength. Therefore, monitoring the gradual increase of light emitted at this wavelength when a ribonuclease is incubated with 6-FAM-dArUdAdA-6-TAMRA allows the quantification of that ribonuclease’s enzymatic activity.

The inhibitory activity of RI and RI variants can be measured using the same assay. The decrease in the rate of the cleavage reaction, due to inhibition of the ribonuclease by sequential additions of RI, causes a detectable reduction in the rate of increase of fluorescence by the cleaved FAM fluorophore, allowing for the calculation of a $K_i$ value. This same assay can be used to quantify the oxidative resistance of the variants of RI that we have created. If the variants of RI that we have created are more

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**Figure 16: A simplified model of Förster resonance energy transfer.** When an isolated fluorophore (A) is excited by light at or near its excitation wavelength (its excitation spectrum), it releases light of a lower energy, or of a longer wavelength, at or near its emission wavelength (its emission spectrum). When the emission spectrum of one fluorophore overlaps with the excitation spectrum of a second fluorophore that is in very close proximity (B), the emission of the excited fluorophore is quenched, as the energy is instead transferred to the second fluorophore. The second fluorophore then releases this energy as light within its own emission spectrum. Excitation and emission wavelength data for FAM from Mineno et al. (231); data for TAMRA is shown as listed by Sigma-Aldrich (232).
oxidation-resistant than wild-type RI, the impact of hydrogen peroxide on the variants’ inhibitory activity should be decreased relative to wild-type RI. Consequently, this assay will allow us to report on both the activity and oxidative resistance of the RI variants we have produced.

*Measuring thermal stability: Is it hot in here, or is it just me?*

Proteins are perhaps the most intricate pieces of chemical origami that exist in the natural world. The present structure of RI in humans is the result of hundreds of millions of years of evolutionary fine-tuning. This configuration is not set in stone; indeed, as already discussed, reactive oxygen species pose a lethal threat to the structure of RI. However, heat can pose a threat to RI as well. At a certain temperature, known as the melting temperature or $T_m$, a protein will succumb to enthalpy and its ordered structure will unfold (198). The more stable the non-covalent interactions are within a folded protein, the higher the $T_m$ value is.

The metamorphosis of a protein structure that occurs at its $T_m$ can be exploited to analyze that protein’s structural stability. The unfolding of a protein leads to the exposure of hydrophobic residues which, in the folded state, are typically buried within its structure, away from the water surrounding it. A number of fluorescent dyes have been discovered whose emission intensifies significantly when bound to the hydrophobic regions of protein; the considerable increase of exposed hydrophobic residues during protein unfolding means that this process can be detected by a concurrent increase in the fluorescence of these dyes (199). This phenomenon has been put to use in several studies to evaluate the stability of proteins (200) (201) (202) (203) (204) (205). The assay which takes advantage of this fact has come to be known
as **differential scanning fluorimetry** and can be performed using a real-time PCR machine, a device normally used to quantify nucleic acids. This screening method will allow us to identify the $T_m$ of each of the RI variants we have created, thereby giving us a measure of the relative stability of the folded structure of each variant.

**Comparing structures using circular dichroism spectroscopy**

For proteins, structure is function. The 3-dimensional placement of the atoms in a protein will directly affect how it binds or interacts with other molecules. Even subtle changes in a protein’s conformation will inevitably modify its activity. While we have altered several of the amino acids in RI, we have done so with the intention to not disrupt its structure. We must find a way to show that we have been successful in this regard or else we cannot associate any of the altered characteristics of our variants exclusively with the substitutions we have made.

One established method to demonstrate the conservation of protein structure is circular dichroism (CD) spectroscopy. CD spectroscopy makes use of circularly polarized light, or light whose electric field vector draws out a helix as it travels through space (Figure 18). Asymmetrical secondary structures such as α-helices and β-strands absorb left- and right-handed circularly polarized light unequally; this phenomenon is known as circular dichroism (206) (207). This differential absorbance can be detected by a spectrophotometer and used to approximate the fraction of a protein that is the aforementioned secondary conformations (208). Changes in the absorbance of circularly polarized light, or the CD spectrum, between a wild-type protein and a variant that has been produced via site-directed mutagenesis are indicative that the mutation has produced a significant change in protein structure (209).
We know that RI now finds itself in the midst of several pathways that are tied to pathologies such as cancer in occasionally paradoxical ways. The evolution of its abundant number of cysteines may have lent RI its fragility, lent it its incredible binding capabilities, both, or neither; how these characteristics play into its biological roles is a mystery. This assay, along with those described above, will allow us to collect important data about the nature of RI and the cysteines it contains.

**MATERIALS AND METHODS**

*General Materials*

All chemicals used were of reagent grade. All plasmids were transformed into and propagated in DH5-alpha *E. coli* (prepared at Lawrence University) grown at 37°C by standard protocol. LB media contained 10g tryptone, 5g yeast extract, and 10g NaCl per liter. TB media contained 12g tryptone, 24g yeast extract, and 4mL glycerol per liter.
with KH$_2$PO$_4$ added to 17mM and K$_2$HPO$_4$ added to 72mM. Plasmids were isolated via miniprep purification (Qiagen; Hilden, Germany) using manufacturer’s guidelines. Precased SDS-PAGE gels were 4-20% (Bio-Rad; Berkeley, CA). Primers were custom-synthesized by Integrated DNA Technologies (Coralville, IA). Sequencing reactions were performed at the DNA Analysis Facility on Science Hill at Yale University (New Haven, CT).

*Development of wild-type RI and RI variant expression constructs*

The original wild-type human RI gene was obtained from the lab of Ronald T. Raines at the University of Wisconsin-Madison. This gene and partially-completed versions of each variant gene were previously cloned into vector pET19-b (EMD Millipore; Billerica, MA).

Poor purification of RI from the pET19-b expression system led us to sub-clone into a maltose binding protein (MBP) fusion vector, pMAL-c5G (New England Biolabs; Boston, MA). Wild-type and variant RI genes were removed from pET19-b+RI via double digestion with BamH1 (Promega; Madison, WI) and Nde1 (New England Biolabs) in solution with BSA and NEBuffer 3 (New England Biolabs; Boston, MA) at 37°C. Restriction enzymes were brought to 5% (v/v) of supplied concentration via repeated addition to reaction mixtures over the course of 3 hours. The destination vector, pMAL-c5G, was digested simultaneously with the same restriction enzymes. Following digestion, the linearized pMAL-c5G was treated with Antarctic phosphatase (New England Biolabs; Boston, MA) in the supplied buffer for 15 minutes at 37°C to prevent auto-ligation.
Digestion reaction mixtures were concentrated down into a small volume using either the DNA Clean & Concentrator-5 or DNA Clean & Concentrator-25 kit (Zymo Research; Irvine, CA). Eluted samples were mixed with DNA loading dye and digested products were then separated via electrophoresis using a 1% agarose gel containing GelRed stain (Biotium; Hayward, CA). Slices of the gel containing bands corresponding to the RI gene (~1500bp) and linearized pMAL-c5G vector (5721bp) were excised using a clean razor blade to isolate the desired digestion products. DNA was purified from the agarose gel using the QIAquick gel extraction kit (Qiagen; Hilden, Germany). Samples were then further concentrated using the DNA Clean & Concentrator-5 kit. DNA concentration was measured using a NanoDrop 2000 UV-visible spectrophotometer (Thermo Fischer Scientific; Waltham, MA).

Ligation reactions were performed using T4 DNA Ligase (Promega; Madison, WI) using LigaFast buffer (Promega; Madison, WI). Reactions were performed using either a 1:1, 1:2 or 1:3 mole-to-mole ratio of linearized vector to RI (or RI variant) gene insert. Ligation reaction was allowed to proceed at room temperature for 15 minutes, after which 1µL of each reaction mixture was transformed into DH5-alpha E. coli. Transformed cells were plated on LB agar with either 200µg/mL ampicillin or 50µg/mL carbenicillin and incubated overnight at 37°C.

DH5-alpha colonies that appeared after overnight incubation were qualitatively screened to check for the presence of the insertion of a gene into pMAL-c5G using PCR. Colonies were resuspended in 75-100µL of double-distilled water; 1µL of this resuspension was added to a 20µL PCR reaction mixture containing GoTaq Flexi DNA polymerase (Promega). This reaction used a forward primer ("pMAL FWD") that binds roughly 100bp upstream of the RI gene and a reverse primer ("pMAL REV") roughly
100bp downstream of the RI gene; both sites are located within the pMAL-c5G vector. Following PCR amplification, reaction products were separated on a 1% agarose gel containing GelRed stain; colonies which contained pMAL-c5G+RI showed a band at roughly 1700bp. Diluted colony samples with positive results from this preliminary test were then used to inoculate 5-10mL of LB media; pMAL-c5G-RI was then isolated from this culture via miniprep purification. Proper insertion of the RI gene was validated by DNA sequencing.

Issues with digestion of the fusion protein expressed from pMAL-c5G-RI led us to sub-clone once again into a new vector, pMAL-c5E, by repeating the above procedure for all seven genes (wild-type plus the six variants).

Mutations and incomplete variants were detected via analysis of the sequence data. The A21/B21, A10, cysteine-free, and non-repeating variants all required repair due to various mutations. Unfortunately, the A17 variant was lost by previous students and will need to be reconstructed. The necessary site-directed mutagenesis was performed using the QuikChange Lightning kit (Agilent; Santa Clara, CA). Briefly, mutagenesis primers were designed to make point mutations at the desired site in each gene while avoiding mis-priming at other locations. Mutagenesis, DpnI digestion of the template DNA, and transformation of mutated plasmid were performed according to the product manual. Correct mutagenesis was validated by DNA sequencing.
Expression and crude purification of RI and RI variants

Preliminary expression protocols were tested and refined by transforming pET19-b-RI into BL21(DE3) E. coli (Lucigen; Middleton, WI). Expression of the RI from pET19-b-RI in BL21(DE3) is under control of the lac repressor. Transformed cells were used to inoculate small (5-10mL LB containing 50µg/mL carbenicillin) starter cultures which were then (while in log-phase growth) used to inoculate 1-liter cultures of LB auto-induction media, which prevents expression from pET19-b-RI until the culture is in log-phase growth (210).

After induction, 1-liter cultures of BL21(DE3) used for RI expression were pelleted via centrifugation at 7000xg for 30 minutes. Cell pellets were resuspended in a minimal volume (5-20mL) of lysis buffer (20mM Tris HCl, pH 8.0; 100mM NaCl; 10mM EDTA; 1mM DTT) and incubated with 0.1mg/mL lysozyme and 1% (v/v) Halt protease inhibitor cocktail (Pierce; Rockford, IL) for 30 minutes at room temperature. Cells were then sonicated (40% power, 3x15 seconds) to complete lysis. Lysate was centrifuged to separate soluble and insoluble fractions for 30-60 minutes at 13,000xg.

RI was isolated from soluble lysate using Ni-NTA resin microcentrifuge spin columns (Qiagen; Hilden, Germany). The resin was pre-equilibrated with 1200µL Ni-NTA binding buffer (50mM NaH₂PO₄, 500mM NaCl, 20mM imidazole, 1mM DTT) Clarified lysate was applied to the column, which was then washed with 1200µL of binding buffer. RI was eluted from this resin using 200µL of binding buffer containing 100mM imidazole.

Expression of wild-type RI as a fusion protein with MBP as the fusion partner was tested and refined in HI-Control 10G, C41(DE3), C41(DE3)-pLysS, C43(DE3), and C43(DE3)-pLysS E. coli strains (Lucigen; Middleton, WI). These cells were transformed
with pMAL-c5G-RI. Strains not containing the pLysS plasmid (which codes for chloramphenicol resistance) were also transformed with pGro7 (Takara; Otsu, Japan), a plasmid coding for GroEL/ES chaperone proteins along with chloramphenicol resistance. Transformed cells were used to inoculate starter cultures (5-10mL LB, 50µL/mL carbenicillin, 25µg/mL chloramphenicol) which were used to inoculate 1 liter of media in a Fernbach flask. Two types of media were tested: TB media and “RI minimal media”, inoculated with bacteria after following the protocol below (Table 4).

| RI Minimal Media | 
|-----------------|-----------------|
| **BEFORE AUToclAVING (PER 1L)** | **AFTER AUToclAVING (PER 1L)** | **Trace Element Solution (per 1L)** |
| Reagent | Amount | Reagent | Amount | Reagent | Amount |
| (NH₄)₂SO₄ | 2.68g | glucose | 10-15g | CaCl₂·2H₂O | 0.50g |
| Na₂SO₄ | 2.00g | 1.0M MgSO₄ | 3mL | ZnSO₄·7H₂O | 0.18g |
| NH₄Cl | 0.50g | 1.0M Thiamine HCl | 100µL | MnSO₄·H₂O | 0.10g |
| K₂HPO₄ | 14.6g | trace element sol’n | 2mL | disodium EDTA | 20.1g |
| NaH₂PO₄·H₂O | 3.60g | carbenicillin | 50mg | FeCl₃·6H₂O | 16.7g |
| (NH₄)₂-H-citrate | 1.00g | chloramphenicol | 25mg | CuSO₄·5H₂O | 0.16g |
| Pure H₂O | to 1.0 L | | | CoCl₂·6H₂O | 0.18g |
| (Adjust pH) | pH 7.00 | | | |

Table 4: Protocol for the preparation of RI minimal media. Trace element solution is prepared separately and should be sterile-filtered before use.

RI minimal media is based on the mineral salt media used by Šiurkus and Neubauer (194). Inoculated TB media and RI minimal media cultures were incubated (37°C, 220rpm) to an OD₆₀₀ of 0.5, as detected by a Varian Cary 50 UV-visible spectrophotometer (Agilent; Santa Clara, CA). At this point, the expression of GroEL/ES was induced by adding 0.4g/liter of L-arabinose. 2 hours following GroEL/ES induction, incubation temperature was reduced to 22°C and the expression of MBP-RI was induced by addition of IPTG (a lactose analog) to a final concentration of 1mM. 2 hours following MBP-RI induction, DTT was added to a final concentration of 12mM.
Cultures were allowed to grow for another 2 hours, at which they were pelleted (7000xg, 30 minutes). SDS-PAGE samples were collected prior to induction of GroEL/ES, prior to induction of RI, and prior to final centrifugation.

Cell pellet was resuspended in 20-40mL (final volume) of lysis buffer and transported on ice to University of Wisconsin-Oshkosh for lysis via a French press. Halt protease inhibitor cocktail or PMSF (a protease inhibitor) was added to each resuspension immediately prior to lysis. Lysate was centrifuged (49,000xg, 60-90 minutes) to separate soluble and insoluble fractions. SDS-PAGE samples were collected from the total lysate (total cell extract) and from the soluble lysate.

Later MBP-RI purification was performed using via FPLC using an MBPTrap 5mL FF column (GE Life Sciences; Fairfield, CT), which binds MBP. FPLC purification was performed at 4°C. The column was pre-equilibrated with 5 CV of amylose binding buffer, after which the clarified lysate was applied. The column was then washed with 8 CV of amylose binding buffer, after which MBP-RI was eluted in 2mL fractions using amylose binding buffer with 25mM maltose. All buffers and samples were applied to the column at a maximum flow rate of 2mL/min. The protein-containing fractions are identified using UV detection of the column eluate and are pooled and stored at 4°C. Before column is used again, it is regenerated by pumping through 5 CV of milliQ water, 5 CV of 0.5M NaOH or 0.1% SDS, and then another 5 CV of milliQ water.

Expression of MBP-RI and MBP-RI variants from the pMAL-c5E vector was performed exclusively using the C41(DE3) strain of E. coli in RI minimal media, using the protocol described above for expression in the pMAL-c5G vector.
**Digestion of MBP-RI and MBP-RI variant fusion proteins**

MBP-RI and MBP-RI variant fusion proteins expressed from the pMAL-c5G-RI vector were digested using Genenase I (New England Biolabs; Boston, MA). The UV$_{280}$ absorbance of pooled MBPTrap elution fractions were determined using a Varian Cary 50 UV-visible spectrophotometer in a 1cm quartz cuvette. Using a theoretical extinction coefficient for the fusion protein computed using the ExPASy ProtParam tool offered by the SIB Swiss Institute of Bioinformatics, absorbance values were converted to protein concentrations using the Beer-Lambert law (211) (212) (213) (214). Genenase I was added to RI at a final concentration of 10 µg Genenase I per milligram of MBP-RI. Time-course trials found that maximum digestion could be attained following a 36 hour incubation at 4°C.

MBP-RI and MBP-RI variant fusion proteins expressed were digested using recombinant light-chain enterokinase provided from two sources (New England Biolabs; Boston, MA & R&D Systems; Minneapolis, MN). Concentrations of MBPTrap eluate samples were measured as described above. Concentrations of 0.0001% (w/w) of New England Biolabs’ enterokinase and 0.005% of R&D Systems’ enterokinase were used for digestions, which were allowed to proceed at room temperature for 60 hours. DTT was added at the beginning of the digestion to a concentration of 10mM.

**Isolation and further purification of RI and RI variants**

Digested MBPTrap eluate samples were diluted using anion exchange equilibration buffer (20mM Tris-HCl, pH 7.5; 10mM DTT; 1mM EDTA) to a volume of 50mL (typically ~1:5 dilution) to reduce the NaCl concentration. Samples were then purified via FPLC at 4°C. 5 CV of anion exchange equilibration buffer was used to pre-
equilibrate a HiTrap Q 5mL HQ column (GE Life Sciences; Fairfield, CT). The digested MBP/RI sample was then applied to the column. The column was washed with 5 CV of equilibration buffer, after which bound protein is eluted using a linear gradient of equilibration buffer starting at 0 and reaching 0.4M NaCl after 20 CV. All buffers and samples were applied to the column at a maximum flow rate of 2mL/min. Eluate was collected in 2mL fractions. Protein-containing fractions were identified by UV absorption and analyzed via SDS-PAGE.

HiTrap Q eluate was further purified to fully isolate RI via gel filtration. Eluate samples were concentrated to a volume of <5mL using 20mL, 9k MWCO Pierce protein concentrator centrifuge tubes (Thermo Scientific; Waltham, MA). Concentrated samples were applied to a poured column of Superdex G75 gel filtration resin (GE Life Sciences; Fairfield, CT) and flowthrough was collected in 5mL fractions. The elution of protein was monitored via UV absorption peaks of the column flowthrough. Protein-containing fractions were analyzed via SDS-PAGE; samples suspected to contain RI were concentrated to a final volume of <5mL once more using 20mL, 9k MWCO Pierce protein concentrator centrifuge tubes. Protein concentration was again detected by UV$_{280}$ absorption and calculated using the Beer-Lambert law.

Differential scanning fluorimetry

$T_m$ was measured in frosted PCR tubes containing a 1:5000 dilution of SYPRO Orange concentrate (Life Technologies; Carlsbad, CA), 10mM HEPES-NaOH, pH 7.5, 150mM NaCl, and 5mM DTT, and 0.1mg/mL RI (or RI variant) in a total volume of 20µL. Samples were placed in an Applied Biosystems 7500 real-time PCR system (Life
Technologies; Carlsbad, CA) and heated from 25°C to 99°C in 0.74°C increments.

Changes in the fluorescence intensity were monitored with the FAM filter.

$T_m$ values for each sample were measured by finding the temperature corresponding to the maximum value of the first derivative of the fluorescence curve, as performed by DeSantis and coworkers (215). First derivative data was reported by the software bundled with the real-time PCR system and exported into Microsoft Excel for precise determination of the minimum point in the data set.

**RNase A inhibition assays using 6-FAM-dArUdAdA-6-TAMRA**

Inhibition assays were performed in a clear 4-sided plastic cuvette containing 3mL of an aqueous solution of 100mM MES-NaOH (pH 6.0), 100mM NaCl, and 5mM DTT along with 0.60µM 6-FAM-dArUdAdA-6-TAMRA (custom-synthesized by Integrated DNA Technologies; Coralville, IA). To each cuvette, a micro stirbar (treated in 10% nitric acid to remove ribonuclease contamination) was added. Cuvettes were placed in a Quantum Master 4 fluorimeter (Photon Technology International; Birmingham, NJ) set to an excitation wavelength of 492nm and a detection wavelength of 515nm (corresponding to the excitation and emission wavelengths of FAM). After the fluorimeter began acquiring data, RNase A was added to a concentration of 25pM. After one minute of steady-state RNase A activity, RI or an RI variant was added in one-minute intervals in increasing concentrations. The effect on RNase A activity was measured as the decrease in average derivative of fluorescence intensity (as reported by the associated software) over each one-minute interval relative to RI concentration.
RESULTS

Expression and purification of RI

Many of the expression and purification systems I tested failed to result in the successful isolation of active RI, although the step of the process at which the failure occurred varied. The expression of 6xHis-RI from the pET19-b vector using LB auto-induction media was successful, but not robust (Figure 19). Purification of 6xHis-RI using Ni-NTA resin yielded only inactive protein. The presence of DTT is necessary in solution with RI to prevent its oxidation; however, DTT also reduces the positively-charged nickel in the resin, causing damage and leading to high amounts of non-specific binding (Figure 20).

Figure 19: Western blot of whole cell lysate. Cultures grown in auto-induction LB media with or without added lactose, which induces expression of 6xHis-RI from pET19-b. “RI Standard” is recombinant RNasin™ ribonuclease inhibitor (Promega; Madison, WI).

Figure 20: Failed purification of 6xHis-RI from BL21(DE3) cells. Arrowheads mark location of 6xHis-RI. “MW Std.” is Precision Plus Protein™ dual color molecular weight standard (Bio-Rad).
The expression of MBP-RI fusion protein from the pMAL-c5G vector was tested in several strains of *E. coli*. HI-Control 10G *E. coli* did not express any detectable amounts of MBP-RI. Expression was then screened in four cell types: C41(DE3), C43(DE3), C41(DE3)-pLysS, and C43(DE3)-pLysS. pLysS-containing cells are engineered to prevent degradation of toxic proteins and cell death due to toxic proteins (216). The C41(DE3) and C43(DE3) cells were also co-transformed with the pGro7 plasmid, which expresses the GroEL/ES chaperone complex. The pGro7 and pLysS plasmids both contain chloramphenicol resistance; therefore, it was not possible to screen for pLysS-containing cells which also were successfully transformed with pGro7. Expression of MBP-RI was apparent in all strains except for C43(DE3)-pLysS (Figure 21). C41(DE3) cells produced the most soluble, active MBP-RI by culture volume, possibly due to the co-expression of GroEL/ES.

![Figure 21: Expression of MBP-RI from pMAL-c5G in C41 and C43 E. coli strains.](image)

3 samples of culture were taken from each strain tested following induction of MBP-RI and GroEL/ES expression. Arrowheads mark location of MBP-RI (~90kDa) and GroEL (~60kDa).
Expression of MBP-RI and GroEL/ES by C41(DE3) cells was tested in two media types: TB media and RI minimal media. TB media contains a large amount (relative to LB) of tryptone and yeast extract, providing crude but plentiful sources of nutrients. RI minimal media contains a variety of essential salts and a limiting carbon source. Although the cultures grown in TB media grew faster, the recovery of soluble MBP-RI was not significantly more fruitful than from RI minimal media, relative to the volume of culture used. The higher cell density of TB media cultures at the end of the expression period also lent itself to more viscous lysate that was considerably more difficult to separate into soluble and insoluble fractions when compared to the RI minimal media cultures. For these reasons, RI minimal media was used for all subsequent expression cultures.

The digestion of the MBP-RI fusion protein expressed from pMAL-c5G was inefficient (Figure 22). MBP fusion proteins expressed from this vector are linked with a peptide containing a site where Genenase I, an engineered protease, can cleave the protein of interest from MBP. Incubations of MBP-RI (crudely purified using an MBPTrap column) with Genenase I resulted in largely undigested protein, even after 60 hours at 4°C or 22°C. These poor results, along with the discontinuation of Genenase I by New England Biolabs, the sole provider, necessitated the use of a new expression vector, pMAL-c5E.
Expression from pMAL-c5E and pMAL-c5G in C41(DE3) cells yielded a similar amount of MBP-RI. Purification of pMAL-c5E from clarified lysate via FPLC using an MBPTrap column resulted in a crude isolation of MBP-RI along with several contaminants, the most abundant of which was a co-expressed chaperone, GroEL (Figure 23, lane B). Digestion of MBP-RI using enterokinase was not complete but was more efficient than digestion by Genenase I (Figure 23, lane C).

Anion exchange chromatography using a HiTrap Q column succeeded in removing many of the unidentified contaminants from the digested crude sample obtained from the MBPTrap column, but did not completely eliminate the contamination of the sample with GroEL (Figure 23, lane D). This purification step also failed in separating RI from MBP. Further purification using size exclusion chromatography succeeded in isolating RI to high purity (Figure 24). However, the yield of recovered RI from this step was minimal.

Purification of the B29 and cysteine-free RI variants has been attempted using this system, with similar results (Figures 23,24). However, both proteins eluted from the size exclusion chromatography column earlier than wild-type RI did, relative to the volume of elution buffer which had been passed through the column. Protein came off at the same point as wild-type RI during the purification of both variants, but SDS-PAGE analysis of the corresponding elution fractions did not reveal the presence of protein. Both variants were also recovered in low yield relative to the quantity purified after anion exchange chromatography.
Figure 23: Crude purification of RI and RI variants using amylose affinity (MBPTrap) and anion exchange (HiTrap Q) chromatography. MBP-RI expressed in C41(DE3) cells in RI minimal media from vector pMAL-c5E. All lanes contain samples derived from cells expressing RI of the type shown above. A lanes are samples of total cell extract. B lanes are samples of the MBPTrap column elution. C lanes are samples of the MBPTrap column elution after digestion with enterokinase. D lanes are samples of the HiTrap Q column elution. “RI Std.” is recombinant RNasin™ ribonuclease inhibitor (Promega).

Figure 24: Purification of RI and RI variants by size exclusion chromatography. Samples of cysteine-free (CF), B29, and wild-type RI taken of elution fractions from a Superdex G75 gel filtration column. RI standard is recombinant RNasin™ ribonuclease inhibitor (Promega).
**Thermal stability of RI and RI variants**

The $T_m$ of human RI has not been previously reported. Using differential scanning fluorimetry, we measured a $T_m$ for wild-type human RI of 49.1°C. The $T_m$ of the B29 variant of RI was not significantly different; however, the $T_m$ of cysteine-free variant was slightly higher than that of wild-type RI.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$T_m$</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type RI</td>
<td>49.1</td>
<td>0.27</td>
</tr>
<tr>
<td>B29 RI</td>
<td>49.2</td>
<td>0.13</td>
</tr>
<tr>
<td>Cysteine-Free RI</td>
<td>*51.1</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*Table 5: Melting temperature of RI and RI variants.* Melting temperatures (±SD) measured using differential scanning fluorimetry performed in triplicate. Melting temperatures were measured as the temperature corresponding to the maximum value of the first derivative of the fluorescence intensity of SYPRO Orange.

*indicates statistically significant difference (p<0.05).

**Inhibitory activity of RI and RI variants**

Qualitative tests of the inhibitory activity of wild-type RI along with the B29 and cysteine-free RI variant were performed using the 6-FAM-dArUdAdA-6-TAMRA substrate. Eluate recovered from anion exchange chromatography for each sample was brought to an equal concentration using anion exchange equilibration buffer. The volume of B29 RI required to cause complete inhibition of RNase A activity was 10-fold that of wild-type RI solution. This suggests that B29 RI has roughly 10% of the inhibitory activity of wild-type human RI. Cysteine-free RI did not cause a detectable reduction in RNase A activity.
DISCUSSION

Expression and purification

Previous studies have made use of the remarkably tight binding interaction between RI and RNase A to aid in the purification of RI (70) (179). RNase A, which is robust and relatively easy to purify in high yields, can be covalently attached to resin beads. When a crude protein sample containing RI is passed through these beads, RI binds to the RNase A and sticks, while all other protein flows through. However, we could not take advantage of this system, as we could not predict whether the RI variants we had created would bind to RNase A. Our efforts to optimize a purification protocol are still ongoing.

Early trials in which 6xHis-RI was expressed from the pET19-b-RI vector gave insight into the multiple hurdles which stand in the way of successfully purifying RI. Expressing high levels of RI, keeping it soluble and folded properly, keeping it in a reduced state, and isolating it from other proteins are all their own separate challenges. Screening various cell strains, media conditions, and growth temperatures led us to high expression levels of RI that could be reproduced from culture to culture. Expressing RI as a fusion protein with MBP and co-expressing with GroEL/ES was vital in increasing the proportion of RI that remained soluble. Adding DTT to the culture media concurrent with RI expression and keeping fresh DTT present at every step during and after lysis has been important in minimizing the loss of RI to oxidation. Even the way in which we lysed cells was evaluated; using a French press has allowed much more reliable lysis of cells and recovery of RI.
The isolation of soluble and active RI remains to be fully optimized. The MBPtrap column has only facilitated the crude purification of RI (Figures 22,23). Much of the contamination, judging by the protein size and abundance, is GroEL. The yield of MBP-RI from the MBPtrap column has been consistent regardless of the amount of crude lysate loaded (data not shown), indicating that we have been attempting to load it past beyond its maximum capacity. In this case, other proteins may be getting caught with MBP-RI in the overloaded column and eluting along with it once the MBP-RI is freed (via the addition of maltose, which competes MBP off the maltose covalently bound to the column resin). Lowering the amount of lysate added at one time may increase the overall yield while reducing the overall contamination of other proteins in the eluate due to non-specific binding.

Another tactic that may reduce the amount of GroEL that is eluted along with MBP-RI is the addition of either an ATPase or additional ATP to the cell lysate prior to purification. ATP triggers multiple changes in the structural conformation of GroEL which, in vivo, allow for the association of GroES and a shift from a hydrophobic protein-binding state to a hydrophilic protein-folding state (217). We cannot be sure which conformation is dominant in our lysate samples but it is possible that increasing or depleting the ATP concentration may discourage the non-specific binding of GroEL within the MBPTrap column.

Anion exchange chromatography using the HiTrap Q column has resulted in only a marginal degree of purification. Proteins elute from an anion exchange column sequentially, based on their isoelectric point (or pI; a determinate of a protein’s net charge at a given pH). Proteins with different isoelectric points elute from the column at different concentrations of NaCl (218) (219). The pI of MBP is 5.1, and pI of GroEL is
4.8; both of these are close to the value of 4.7 for RI, which jeopardizes our efforts to purify RI from MBP using this method (220) (221). This may explain why both proteins have eluted along with RI from the HiTrap Q column simultaneously (Figure 23); however, this has not always occurred (Figure 22). It appears that lower contamination in the sample eluted from the MBPTrap column lends itself to lower contamination from the HiTrap Q column. Perhaps the solutions for decreasing the non-specific binding during the MBPTrap purification described above will be sufficient to solve this problem. If not, other purification options are available, such as hydrophobic interaction chromatography, which separates proteins based on their exposure of hydrophobic amino acids rather than their isoelectric point (222).

Faced with the failure of anion exchange chromatography to completely purify RI from MBP once digested with enterokinase, we turned to size-exclusion chromatography. This led to a large loss in overall protein yield. It is our hope that we can avoid the time-intensive process of size-exclusion chromatography all together once we optimize the prior two steps of RI purification.

_Termal stability and inhibitory activity of RI variants_

In replacing the cysteines of RI in various ways, we sought to answer a basic question about this protein: have these cysteines been selected for because they play an important structural role, because they are necessary for binding ribonucleases, or because it was important that RI be sensitive to oxidation? Although our results so far in characterizing our RI variants are still preliminary, we can still make some interesting (although equally preliminary) conclusions in this regard.
The conservative substitution of various similar amino acids in human RI does not decrease the structural stability of the protein. Five cysteines are replaced in the B29 variant of RI, all in a conserved position throughout the repeating structural modules. However, these replacements did not change the $T_m$ of the B29 variant in a significant way (Table 5). Furthermore, the total replacement of every cysteine in RI led to an increase in overall structural stability, as measured by a significant increase in the $T_m$. It is important to note here that CD spectroscopy will be necessary to demonstrate that this variant still exists in the same tertiary structure as wild-type RI. However, if it does, we have shown that the presence of cysteine in RI serves to decrease the overall structural stability.

By its design, the cysteine-free RI variant is not susceptible to cysteine-mediated oxidation. At the same time, however, ribonucleases are not susceptible to cysteine-free RI, as we have been unable to detect that this variant has any inhibitory activity. While we have not yet assessed the oxidative sensitivity of the B29 variant, it nevertheless shows reduced inhibitory activity against ribonucleases (relative to wild-type RI) after the substitution of an intermediate amount of cysteines.

Based on these findings, we propose that the susceptibility of RI to oxidation is an integral part of its biological function. If it is the same residues, the cysteines, which are necessary for inhibitory activity and oxidation sensitivity, it is possible that these residues have been selected for and preserved in order to maintain both phenotypes simultaneously. At least two of these cysteines are known to be important in non-covalent interactions with ribonucleases, but they also may contribute to binding in another fashion. RI relaxes its structure and stretches open when it binds to ribonucleases (72). If this stretching is necessary to accommodate binding, then the
cysteine-mediated structural destabilization we have identified may be necessary for the activity of RI. Variants stabilized by the removal of cysteine may be too rigid to “make room” for ribonucleases to bind.

The importance of simultaneously maintaining both of these traits is not clear in either the “intracellular sentry” or anti-oxidant theories of the biological purpose of RI (129) (144). However, in light of the recent discovery of its role in a cellular stress pathway in which the oxidation of RI is necessary to allow for the free activity of angiogenin, such a relationship appears to exist (Figure 25) (147). If the oxidation sensitivity of RI goes hand-in-hand with its ability to modulate ribonucleases, and this interaction is essential, then it is also possible that ribonucleases which are better-inhibited by an oxidation-sensitive RI have co-evolved with RI.

Fully-active variants of RI with reduced sensitivity to oxidation have been created in the past by substituting alanine for cysteine (102). However, the only cysteines that were successfully replaced in such a way were cysteines 328 and 329, which are

Perhaps oxidation sensitivity has evolved to allow RI to be rapidly inactivated...

...allowing vertebrate-specific ribonuclease activity, which responds to oxidative stress

Figure 25: A hypothesis for the evolutionary purpose of rapid oxidation-induced denaturation and inactivation in human RI.
adjacent to each other. Only two pairs of adjacent cysteines exist in human RI (Figure 12), and it is possible that these pairs contribute more strongly to the oxidation sensitivity of RI than to the inhibitory activity of RI. The proximity of the thiol groups in these adjacent pairs makes it more likely for them to form a disulfide bridge, one which creates an eight-membered ring which destabilizes the local structure (102). Importantly, the variants produced by the substitution of this adjacent pair were not completely immune to oxidation. Furthermore, porcine RI has only one such pair, and rat RI does not contain any adjacent cysteine residues (107) (223). For these reasons, it is possible that these adjacent pairs of cysteines have arisen recently in the human lineage and provide added oxidation sensitivity to what is already an oxidation-sensitive protein.

The further development of this hypothesis will require us to complete the expression, purification, and characterization of all six RI variants. As we move towards this goal, we also find ourselves completing a project which may serve as a hub to begin other investigations into the biological properties of RI. If a variant of RI is successfully produced that has reduced sensitivity to oxidation yet maintains its inhibitory activity, the pathology of its substitution for wild-type RI in vivo could allow us to identify the exact processes in which RI is important. Another interesting route to explore is whether or not any of our variants maintain affinity for the anti-tumor protein, PTEN. Does inhibitory activity against ribonucleases or oxidation sensitivity correlate in any way to the ability to interact with this important protein? Changes in the ability of our variants to interact with Drosha could also provide meaningful insights into how RI modulates microRNA processing. Last, but not least, the maintenance of tertiary structure in cysteine-free RI would suggest we have created a highly oxidation-resistant,
stable, and modular scaffold for protein engineering. All of these possibilities keep us excited and driven to continue working to discover more about the role of RI in the chaotic and amazing biochemical realm of life.

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