Effects of RNase L Mutations Associated with Prostate Cancer on Apoptosis
Induced by 2′,5′-Oligoadenylates 1


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ABSTRACT

The RNASEL gene, a strong candidate for the hereditary prostate cancer 1 allele (HPC1), encodes a single-stranded specific endonuclease involved in the antiviral actions of IFNs. RNase L is activated enzymatically after binding to unusual 5′-phosphorylated, 2′,5′-linked oligoadenylates (2–5A). Bioactive phosphorothioate analogues of 2–5A were synthesized chemically and used to study the effects of naturally occurring mutations and polymorphisms in RNASEL. The 2–5A analogues induced RNase L activity and caused apoptosis in cultures of late-stage, metastatic human prostate cancer cell lines DU145, PC3, and LNCaP. However, DU145 and PC3 cells were more sensitive to 2–5A than LNCaP cells, which are heterozygous for an inactivating deletion mutation in RNase L. The RNase activities of missense variants of human RNase L were compared after expression in a mouse RNase L–/- cell line. Several variants (G598, I197L, I220V, G296V, S322F, Y529C, and D541E) produced similar levels of RNase L activity as wild-type enzyme. In contrast, the R462Q (G59S, I97L, I220V, G296V, S322F, Y529C, and D541E) produced similar RNase L activity and caused apoptosis in cultures of late-stage, metastatic human prostate cancer cases. The only known nucleic acid with two or more consecutive 2′,5′ linkages is a unique place in nucleic acid biology by providing an unambiguous signal to RNase L, resulting in cleavage of single-stranded RNA. A large number of 2–5A analogues have been described previously with modifications of the bases, riboses, internucleotide linkages, 5′-phosphoryl groups, and 2′,3′ termini (reviewed in Ref. 5). Nevertheless, development of bioactive and potent small molecule activators of RNase L that are convenient to synthesize and purify have remained elusive. Natural 2–5A molecules are very efficiently degraded by a combination of 5′-phosphatase and 2′,5′-phosphodiesterase activities present in cells and in serum. Therefore, stabilization of the 2–5A molecule to catalyzeable enzymes while retaining RNase L activation ability was an important goal in the present study.

RNase L, the target of 2–5A, is a 741-amino acid protein (human form) with a bipartite domain structure in which the N-terminal half represses the RNase domain in the COOH-terminal region (Fig. 1; Ref. 8). The repressor half consists of nine ankyrin repeats. 2–5A also contains several protein kinase-like domains in its COOH-terminal half. The kinase-like and RNase domains of RNase L are homologous to the Irel proteins, with both kinase and endoribonuclease activities, that function in the unfolded protein response (9).

Observed mutations of RNASEL in men with prostate cancer include: 3G→A, resulting in a methionine to isoleucine missense mutation in the translational start codon, thus preventing translation (1); 795G→T, converting a glutamic acid within the 2–5A binding domain of RNase L to a stop codon (E265X; Refs. 1 and 11); and 471ΔAAAG, causing a frameshift at codon 157 and a translation stop after seven additional codons (Refs. 12 and 13; Fig. 1). Loss of the wild-type RNASEL allele in prostate tumor tissue was reported in cases with either the 795G→T (E265X) or 471ΔAAAG mutation (1, 12). In addition, there exists among prostate cancer cases and controls several missense variants in RNase L (Fig. 1; Refs. 1, 13, and 14–16). Among these, only R462Q has been thus far associated with increased prostate cancer risk (11, 15). For example, we recently implicated the R462Q variant of RNASEL (1385G→A) in unselected (including both familial and nonfamilial) prostate cancer cases (15).

An expanded study was performed on DNA isolated from 423 prostate cancer cases and 454 unaffected sibling controls. A significant association of the R462Q variant with cases was observed (P = 0.011). The odds ratios indicated that carrying one copy of the R462Q variant

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6795
gene increased the risk of prostate cancer by \( \sim 1.5 \)-fold, whereas having two variant alleles doubled the risk. Results implicated R462Q in up to 13% of cases, which if confirmed would make it the most prevalent genetic marker for prostate cancer. Therefore, R462Q could be an important risk marker for prostate cancer in the general male population.

Here, we have investigated the impact of RNase L activated by novel 2'-5A analogues on apoptosis of the human prostate cancer cell lines PC3, DU145, and LNCaP, derived from metastases of bone, bone, and lymph node, respectively. Results show that 2'-5A analogues have the ability to induce apoptosis of such late-stage prostate cancer cells. In addition, among eight naturally occurring missense variants evaluated, only R462Q significantly decreased RNase L activity. The deficiency in the R462Q variant was correlated to a decrease in enzyme dimerization to the active form and a reduction in the ability to cause apoptosis. Our findings support the association of RNase L-R462Q with prostate cancer risk while also highlighting the ability of RNase L activators to induce death of prostate cancer cells derived from late-stage tumors.

**MATERIALS AND METHODS**

**Reagents.** Syntheses of the 2',5'-oligoadenylate moieties were performed using 5'-O-dimethoxytrityl-N'-benzoyl-3'-O-butyldimethylsilyleladenosine-2',5'-diisopropylcyanoethyloxyphosphoramidite (ChemGenes, Ashland, MA). The phosphorylation reagent for the 5' terminus of the 2',5'-oligoadenylate was 2-[2-O-(4,4'-dimethoxytrityl)sulfonyl]ethyl-2,4'-cyanoethyl-5'-diisopropylphosphoramidite (Glen Research, Sterling, VA). Control pore glass supports and the sulfurizing reagent, used at 0.05 m, were also from Glen Research. Other phosphoramidites used were: 5'-O-dimethoxytrityl-N'-benzoyl-2'-O-butyldimethylsilyleadenosine-3'-[2-(cyanoethyl-N,N-diisopropyl)phosphoramidite, 5'-O-dimethoxytrityl-N'-benzoyl-2'-O-butyldimethylsilylecytidine-3'-[2-(cyanoethyl-N,N-diisopropyl)phosphoramidite, 5'-O-dimethoxytrityl-2'-O-butyldimethylsilylethidiumidine-3'-(2'-cyanoethyl-N,N-diisopropyl)phosphoramidite, and 5'-O-dimethoxytrityl-2'-deoxythymidine-3'-[2-(cyanoethyl-N,N-diisopropyl)phosphoramidite (Glen Research).

**Oligonucleotide Synthesis.** Chemical syntheses of the oligonucleotides were performed on 1-μmol columns (Glen Research) using an ABI model 380B automated DNA synthesizer (Applied Biosystems). After syntheses, the columns were removed from the instrument and the supports were transferred to screw-capped vials (4 ml; Wheaton, Millville, NJ). The supports were treated with 3 ml of ammonium hydroxide/ethanol solution (3:1, v/v) for 2 h at room temperature and then at 55°C for 8 h. The solutions were dried in vacuo on a Savant Speed-Vac. The residues were treated with 1 m tetrabutylammonium fluoride in tetrahydrofuran solution (Aldrich, St. Louis, MO) overnight. Tetrahydrofuran was removed using Speed-Vac. Compounds were dissolved in 1 ml of water and vortex mixed. The crude products were purified by HPLC\(^{4}\) on a polystyrene reverse phase column (PPR-100; Hamilton Co., Reno, NV). Solvent A was 10 mm tetramethylammonium dihydrogenophosphate (pH 7.5) in water, and solvent B was 10 mm tetrabutylammonium dihydrogenophosphate (pH 7.5) in acetonitrile:water (8:2, v/v). Elution was with a convex gradient of 5–80% solvent B in solvent A in 60 min at a flow rate of 1.5 ml/min. Fractions containing the compounds were pooled, dried, and then desalted on Sep-Pak columns (Waters Corp., Milford, MA), using 80% methanol as eluent. The tetrabutylammonium salt was transformed into the sodium ion by ion-exchange using Dowex 50W (sodium form; Bio-Rad, Hercules, CA).

Natural 2'-5A \( [\cdot (A'2'P)n]_A \), where \( n = 1 \pm 3 \) was prepared enzymatically from ATP using hexaethylene-tagged and -purified recombinant porcine MN, 42,000 2'-5A synthetase (a gift from R. Hartmann, Cleveland Clinic, Cleveland, OH; Ref. 17). Individual 2'-5A oligomers were purified using reverse-phase HPLC.

**Determination of the Stability of the 2'-5A Analogues in Human Serum.** The stability of 2'-5A compounds (each at 47.8 μM) was determined by incubation in 700 μl of human serum (Sigma-Aldrich, St. Louis, MO), diluted to 800 μl with water at 37°C. Aliquots (100 μl) were removed at 0, 1, 2, 4, 7, and 24 h, heated to 100°C for 5 min, centrifuged 10 min at 10,000 × g at 2–4°C, and the supernatant removed for HPLC analysis. 2'-5A compounds and their degradation products were analyzed by HPLC on an Ultrasphere ODS column (4.6 × 250 mm) (Beckman).

**Cell Culture and Transfections.** Hey1B (human ovarian carcinoma), DU145, PC3, and LNCaP cells were grown in RPMI 1640 supplemented with streptomycin-penicillin and 10% heat-inactivated fetal bovine serum. L929 (mouse fibroblast) and HeLa M cells (human cervical epithelial cells) were grown in DMEM supplemented with streptomycin-penicillin and 10% heat-inactivated fetal bovine serum. Mouse JM3 cells were isolated from a spontaneous rhabdomyosarcoma from RNase L−/− p53−/− mice.\(^{4}\)

Transfection of 2'-5A compounds was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Briefly, cells were plated 1 day before transfection, so that the cells are ∼70% confluent at the time of transfection. 2'-5A was diluted into Optima media (Invitrogen) and then mixed with Lipofectamine 2000 for ∼20 min. The mixture was added to the cells, incubated at 37°C for 3–5 h, then the media was replaced with complete media plus serum.

**Site-directed Mutagenesis of RNase L.** The RNase L point mutants were constructed using the QuickChange XL Site-Directed Mutagenesis Kit from Stratagene. Briefly, a full-length coding sequence DNA for human RNase L in pGEX-4T-3 vector (8) was used as template for mutagenesis. The template plasmid was denatured and annealed with the two synthetic oligonucleotide primers containing the desired mutations. Using the nonstrand-displacing action of Pfu-Turbo DNA polymerase, we extended and incorporated the mutagenic primers, resulting in nicked circular strands. The methylated, non-mutated parental DNA template was digested with DpnI. The circular, nicked double-stranded DNA was transformed into XL10-Gold ultracompentent cells (Stratagene). After transformation, the XL10-Gold ultracompentent cells repair the nicks in the mutated plasmid. All mutants were confirmed by DNA sequencing analysis.

**Expression and Purification of GST-RNase L Fusion Proteins.** Briefly, the cDNAs for wild-type or mutant forms of RNase L in plasmid pGEX-4T-3 were transformed into Escherichia coli DH5α (8). The transformed bacteria were grown at 37°C to an \( \text{OD}_{595} = 0.5 \) before being induced with 0.1 mm isopropyl-1-thio-β-D-galactoside for 3 h. Harvested cell pellets were washed with PBS and resuspended in PBS-C (8). The suspended cells were sonicated on ice, and Triton X-100 was added to a final concentration of 1% (v/v). The supernatants were collected after centrifugation at 16,700 × g for 20 min at 4°C. Purification of fusion proteins were performed as described by the manufacturer of glutathione-Sepharose 4B (Pharmacia).

**Activation of Purified Recombinant RNase L.** Substrate for in vitro RNase L assays was C\(_{51}\)UU\(_{12}\) (prepared on an ABI DNA synthesizer and purified as described above), labeled at its 3' terminus with [\( ^{32} \)P]pCp (300 Ci/m mole; DuPont/New England Nuclear) with T4 RNA ligase (Life Technologies, Inc.; Ref. 18). Briefly, 0.1 μg of purified GST-RNase L (wild type or R462Q) was incubated in the presence or absence of 0.1, 1, 10, and 100 nm 2'-5A analogue on ice for 30 min. Reaction mixtures were incubated further with 80 nm C\(_{51}\)UU\(_{12}\) for 30 min at 30°C. RNA was analyzed in
sequencing gels to measure the extent of RNA degradation. The ratios of degraded RNA substrate to the intact RNA was quantitated in a phosphorimager.

Monitoring RNase L-mediated rRNA Cleavages in Intact Cells. The cell-based RNase L assay was performed as described previously (1). Briefly, cells were transfected with 2–5A using Lipofectamine 2000 (Invitrogen) at the concentrations indicated in the figure legends. After a 3- to 5-h incubation, media were removed and cells were washed twice in 5 ml of PBS. Total RNA was isolated from transfected cells using the Trizol reagent according to manufacturer’s protocol and was quantitated by measuring absorbance at 260 nm. RNA (1 μg) was separated on RNA chips and analyzed with an Agilent Bioanalyzer 2100 (Agilent Technologies). The peak areas of 28S and 18S rRNA and their main cleavage products were measured using the Bio Sizing program (version A.02.01 S1232).

Cell Viability Assays. The viability of cells was determined using the colorimetric CellTiter 96 Aqueous Cell Proliferation Assay, as described (19). Briefly, cells were seeded in 96-well culture plate (5 × 10^3 cells per well) and transfected with various forms of 2–5A at different concentrations. At 18 h after transfection, 50 μl of CellTiter 96 Aqueous reagent (40% v/v dilution in PBS) were added to each well. Plates were incubated at 37°C for 3 h, and absorbance was measured at 490 nm with a 96-well plate reader (model Spectra Max 340; Molecular Devices, Menlo Park, CA).

Western Blots. Protein (100 μg) in cell extracts were separated in 10% polyacrylamide/SDS gels for detection of RNase L, PARP, or β-actin or 12% polyacrylamide/SDS gels for caspase 3. The proteins were transferred to Immobilon-P membrane (Millipore), incubated with monoclonal antibody to human RNase L (20), or polyclonal antibody to human cleaved caspase 3 (Chemicon) or polyclonal antibody to PARP (Cell Signaling), or monoclonal antibody to β-actin (Sigma) for 1 h. Membranes were washed with PBS with 1% Tween 20 and incubated with goat antimouse or goat antirabbit antibody tagged with horseradish peroxidase (Life Technologies, Inc.) for 1 h. Proteins in the blots were detected by enhanced chemiluminescence (Amer sham).

Binding of 2′,5′-Oligoxygenylates to RNase L. A 32P-labeled and bromine-substituted 2–5A analogue, p(A2)p(5)br2A2)5A3[32P]pCP (probe), was cross-linked to GST-RNase L (human; 0.4 μg) or to RNase L in crude cell extracts (200 μg) under UV light, as described (21). The RNase L fusion proteins or cell extracts were incubated with the probe, 10 ng (specificity, 3000 Ci/nmol), in 50 μl of buffer on ice for 60 min. Samples were exposed to 308-nm light to induce covalent cross-linking to RNase L on ice for an antibody to human RNase L (20), or polyclonal antibody to human cleaved caspase 3 (Sigma) on ice for 1 h. BSA (250 μg) and glutathione sepharose 4B was added, and the mixtures were incubated with NT NT NT NT NT 1.24

Table 1 Analysis of 2–5A analogues

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical structure</th>
<th>2-5A Binding activity</th>
<th>RNase L activity in vitro</th>
<th>Stability in human serum (T1/2, h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Human cells</td>
<td>Mouse cells</td>
</tr>
<tr>
<td>Natural phosphodiester 2–5A</td>
<td></td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>1</td>
<td>ppp5′A(2′p5′A)2</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>ppp5′A(2′p5′A)3</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>p5′A2p5′A5</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Phosphorothioate mixed isomer (R5 + S5)</td>
<td>p5′A(2′p5′A)3</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>p5′A(2′p5′A)3</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>Phosphorothioate mixed isomer (R5 + S5) with 2′3′-terminal modification</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>p5′A(2′p5′A)2′-OH2</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>p5′A2p5′A3′p5′A2′OH2</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>p5′A2p5′A3′p5′A2′OH2</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>p5′A2p5′A3′p5′A2′OH2</td>
<td>++</td>
<td>++</td>
<td>NT</td>
</tr>
<tr>
<td>a</td>
<td>Competitive inhibition of binding to GST-RNase L to radiolabeled 2–5A probe at: 1 nM, ++; 10 nM, ++; 100 nM, ++; none at 100 nM, −</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b</td>
<td>Assayed with recombinant RNase L against C3H10T1/2, active [&gt;50% cleavage of RNA] at: 0.1 nM, ++; 1 nM, ++; 10 nM, ++; 100 nM, ++; none at 100 nM, −</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c</td>
<td>Assayed in human HeyIb cells or mouse L929 cells, 1 μl compound transfected for 3 h, percentage rRNA cleavage: 70–80%, +++; 60–70%, ++; 50–60%, ++; &lt;50%, +; none, −; NT, not tested.</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

RESULTS

Activities of Novel 2–5A Analogues. In nature, 2–5A is a short-lived, intracellular mediator of RNase L activity. To evaluate the effects of RNase L within intact cells, we synthesized a series of biostable 2–5A analogues (Table 1). Natural 5′-triphosphorylated 2–5A trimer and dimer (compounds 1 and 2) were synthesized enzymatically from ATP using 2–5A synthetase, whereas a 5′-monophosphoryl phosphodiester trimer (compound 3) and all other compounds were synthesized chemically (see “Materials and Methods”). Compounds 4 and 7 with 5′-ribohosphates and mixed isomer PS linkages had half-lives (t1/2) of >24 h at 37°C in human serum, compared with a t1/2 of only 1.2 h for a phosphodiester form of 2–5A (compound 3; Table 1). Compounds 5–8 have 2′-terminal modifications designed for added stability, including a 2′-O-methyl group (compound 5), and 2′ to 3′ inverted deoxynucleotides (compounds 6–8). The PS derivatives of 2–5A (compounds 4, 5, 7, and 8) were shown to efficiently bind and activate RNase L in a cell-free system and in intact mouse fibroblasts (L929) and/or human ovarian carcinoma (HeyIb) cells. As controls for nonspecific effects, compounds 2 and 6 lack the ability to activate RNase L because they contain only two instead of the requisite three consecutive 2′,5′-linked adenylyl residues.

RNase L Activation by 2–5A Analogues in Prostate Cancer Cell Lines Results in Apoptosis. The effects of the 2–5A analogues on RNase L activity and cell viability were determined in the human prostate cancer cell lines PC3, DU145, and LNCaP. The complete coding sequences of RNase L in these cell lines was determined (Table 2). LNCaP cells are heterozygous for a deletion mutation (471ΔAAAG), causing a frameshift and stop codon (12). In addition, the LNCaP cells are heterozygous for R462Q and D541E missense variants. RNase L in PC3 cells is homozygous for the wild-type accession sequence (NM021133), whereas in DU145 cells are heterozygous for a G296V missense variant of RNase L. Effects on cell viability of transfecting 2–5A compounds were determined by tetrazolium conversion (3–(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfonly)-2H-tetrazolium) assays. A PS 2–5A (compound 4) had a 10-fold enhanced anticalcellular effect on the DU145 cells compared with a natural phosphodiester (compound 1) (Fig. 2). The higher activity of compound 4 is likely caused by its enhanced bioavailability. In contrast, control diadenylate phosphodiester and phosphorothioate compounds that do not activate RNase L (comp-
Table 2 Coding sequence polymorphisms in RNASEL of LNCaP, DU145, and PC3 cell^a^ 

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Exon</th>
<th>Nucleotide alteration</th>
<th>Amino acid change</th>
<th>Heterozygous (Het)/ homozygous (Homo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC3</td>
<td>7</td>
<td>A2172G</td>
<td>None</td>
<td>Homo</td>
</tr>
<tr>
<td>DU145</td>
<td>2</td>
<td>G878T</td>
<td>G296V</td>
<td>Het</td>
</tr>
<tr>
<td>LNCaP</td>
<td>7</td>
<td>A2172G</td>
<td>None</td>
<td>Homo</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>T255C</td>
<td>None</td>
<td>Het</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>471ΔAAAG</td>
<td>Frameshift codon 157/stop codon</td>
<td>Het</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>G1385A</td>
<td>R462Q</td>
<td>Het</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>T1623G</td>
<td>D541E</td>
<td>Het</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>A2172G</td>
<td>None</td>
<td>Homo</td>
</tr>
</tbody>
</table>

^a^ Sequence variations are compared against accession no. NM021133 as “wild type.”

Fig. 2. 2-5A activation of RNase L suppresses DU145 cell viability. DU145 cells were mock transfected or transfected with 2-5A (compounds 1, 4, 2, and 6) at different concentrations, as indicated. At 18 h after transfection, cell viability was determined with a relatively high concentration (50 μM) of a natural 2-5A mixture (see “Materials and Methods”). RNAse L activity was measured from levels of intact 28S and 18S rRNA and specific rRNA cleavage products on RNA chips (1). Because the RNAse L variants were expressed to different levels, rRNA cleavage levels were normalized by 46% at 3 days. These findings demonstrate that the 2–5A analogues can induce apoptosis and/or significantly reduce proliferation of late-stage, metastatic prostate cancer cells when at least one active RNASEL allele is present.

RNAse L^H9262^M Has Reduced RNAse Activity in Comparison with Several Other Missense Variants of RNAse L. Genetic studies have identified several missense variants of RNAse L (Fig. 1; Refs. 1, 11, and 14–16). To determine the effects of the missense mutations, the variants were expressed individually in the mouse RNAse L^−/−^ rhabdomyosarcoma cell line JM03 (see “Materials and Methods”). Expressed levels of RNAse L were measured in protein blots probed with monoclonal antibody against RNAse L (Ref. 20; Fig. 6A). At 24 h after transfection of the RNAse L cDNAs, cells were transfected with a relatively high concentration (50 μM) of a natural 2–5A mixture (see “Materials and Methods”). RNAse L activity was measured from levels of intact 28S and 18S rRNA and specific rRNA cleavage products on RNA chips (1).

Fig. 3. Comparison of RNAse L activity in intact DU145, PC3, and LNCaP cells. Cells were mock transfected or transfected with 2–5A (compound 4) at 0.25 μM or 1 μM. RNA was extracted 5 h after transfection, separated on RNA chips, and analyzed with an Agilent Bioanalyzer 2100. The percentage of rRNA cleavage was determined and indicated.

Fig. 4. Apoptosis as measured by PARP and caspase 3 cleavage in comparison with RNAse L levels in prostate cancer cells treated with 2–5A. DU145, PC3, and LNCaP cells were mock transfected or transfected with natural 2–5A mixture at 10 μM. At 18 h after transfection, cell extracts were prepared and proteins were separated by SDS-PAGE, transferred to Immobilon-P membrane, and probed with antibodies for intact and cleaved PARP (A), cleaved caspase-3 (B), RNAse L (C), and β-actin (D).
incubations, low concentrations (0.1 µM) of a phosphodiester 2′-5′A (compound 3) were used. RNase L cleaves the substrate on the 3′ side of the UU dinucleotide sequence, leaving a 3′-phosphate. The R462Q variant had a ~3-fold reduced rate of RNA cleavage compared with wild-type RNase L, consistent with our previous findings (15). To investigate the cause of the deficiency, 2′-5′A-binding activity was measured by covalent cross-linking of a radioactively labeled, bromine-substituted 2′-5′A analogue under UV light (21). However, the R462Q and wild-type enzymes had equivalent levels of 2′-5′A binding activity (Fig. 8).

RNase L dimerization in response to 2′-5′A binding is necessary for enzyme activation (20). To measure dimerization, GST fusion proteins of the wild-type RNase L or RNase LR462Q were incubated with untagged wild-type RNase L in the absence or presence of 2′-5′A. Dimerization was measured after immobilizing the complex on glutathione-Sepharose. At 0.1, 0.3, and 0.9 µM 2′-5′A (compound 3), RNase L R462Q dimerized to 25, 47, and 64% the level observed with to the level of RNase L that was expressed (Fig. 6A). Most of the variants had levels of RNase L activity that was similar to the wild-type enzyme. The Y529C variant was reduced by ~35%, whereas the other variants had slightly less activity (~20%) than the wild-type enzyme. In contrast, the RNase L R462Q variant was reduced in activity by ~3-fold compared with wild-type RNase L (Fig. 6B).

To analyze further the defect, the wild-type enzyme and R462Q variant were produced as GST fusion proteins in E. coli and after purification were incubated with the synthetic RNA substrate C7 U2 C12-32Pcp in the presence or absence of 2′-5′A (Fig. 7). Because these assays were performed in vitro for relatively brief

Fig. 5. Comparison of the ability of 2′-5′A to suppress viability of different prostate cancer cell lines. DU145, PC3, and LNCaP cells were mock transfected or transfected with 2′-5′A compound 4 (3 µM) or compound 6 (10 µM) every 24 h for 3 days. Cell viability was determined daily with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2H-tetrazolium reagent by measuring the absorbance at 490 nm. Analyses were performed in triplicate, and SDs were calculated.

Fig. 6. Comparison of wild-type and missense variants expressed in RNase L−/− cells. Mouse RNase L−/− JM03 cells were transfected with cDNAs for wild-type or missense mutant forms of RNase L. Cells were transfected for 5 h with a natural mixture of 2′-5′A at 50 µM. A, proteins were separated by SDS-PAGE, transferred to Immobilon-P membrane, and probed for RNase L and β-actin. B, RNA was separated on RNA chips with an Agilent Bioanalyzer 2100. RNase L activity was determined as percentage of rRNA cleavage normalized to the amount of RNase L present.

Fig. 7. Catalytic rate of RNase L R462Q is reduced compared with wild-type RNase L. GST fusion proteins of wild-type and R462Q forms of RNase L (100 ng) were incubated with or without 0.1 µM 2′-5′A (compound 3) on ice for 30 min, followed by incubation with C7UC12-32Pcp at 30°C for 30 min. The RNA was separated in 20% acrylamide sequencing gels. A, autoradiogram of gel. B, phosphorimager quantitation of the percentage RNA cleavage.

Fig. 8. 2′-5′A-binding activity of wild-type and mutant RNase L. A 32P-labeled 2′-5′A analogue was cross-linked covalently to the proteins under UV light. The proteins were separated on a 10% acrylamide gel. An autoradiograph of the dried gel is shown. Wild-type, untagged recombinant RNase L produced in insect cells was used as positive control (Lane 1) in comparison with the GST fusion proteins of wild-type RNase L and RNase LR462Q (Lanes 2 and 3).
wild-type RNase L (Fig. 9). Results indicate that the R462Q enzyme had a reduced capacity to dimerize into its activated state.

The R462Q Variant of RNase L Is Deficient in the Ability to Induce Apoptosis. To determine the effect of the R462Q variant on the ability to induce apoptosis, the wild-type and variant forms of RNase L were expressed in RNase L-deficient HeLa M cells. Subsequently, the cells were mock-transfected or transfected with 2–5A (compounds 4, 5, 7, and 8). Apoptosis was measured by the appearance of cleaved PARP and cleaved caspase 3 (Fig. 10). As a positive control, cleavage of both PARP and caspase 3 were observed in cells treated with tumor necrosis factor α and actinomycin D (Fig. 10, Lane 3). The 2–5A analogues did not induce cleavage of either PARP or caspase 3 in cells transfected with the empty vector (Fig. 10, Lanes 4–8). In contrast, cleavage of both PARP and caspase 3 was observed in cells expressing wild-type RNase L (Fig. 10, Lanes 10–13). PARP cleavage was reduced ~2-fold in cells expressing RNase L>R462Q; whereas caspase 3 cleavage was undetectable, despite expression of similar levels of RNase L and RNase L>R462Q. These findings suggest that the association of RNase L>R462Q with prostate cancer risk (11, 15) correlates with deficient enzymatic activity and consequently in a decreased ability to induce apoptosis.

DISCUSSION

Probing the Tumor Suppressor Role of RNase L with Novel Biostable 2–5A Analogues. RNase L was suggested to function as a tumor suppressor based on its mapping to the HPC1 allele and its role in mediating apoptosis in response to several types of external stimuli (reviewed in Ref. 2). To efficiently activate RNase L in intact mammalian cells, we synthesized a series of novel, biostable analogues of 2–5A. PS derivatives of 2’,5’–linked oligoadenylates, in which sulfur atoms replace nonbridging oxygen atoms, were reported previously to be resistant to degradation in serum, particularly those with S₅P stereocoreconfigurations (23). PS-linked 2–5A can either activate or inhibit RNase L, depending on the chirality of the derivatives (24). R₅P PS linkages in 2’,5’–triadenylates are activating, whereas the S₅P derivatives are inhibitory. In addition, phosphorothioate derivatives of 2–5A were reduced in their ability to activate RNase L by a factor of 10 (25). Surprisingly, we observed that mixed isomer (R₅P + S₅P) PS derivatives of 2’,5’–oligoadenylates with 5’–thiophosphoryl groups with and without 2’,3’ modifications are both biostable and able to potentially activate RNase L (Table 1). For example, the PS, biostable 2–5A analogue ps5’A(2’ps5’A)₃ (compound 4) had an IC₅₀ of 0.4 μM (concentration required to inhibit cell viability by 50%) in DU145 cells. In contrast, the IC₅₀ of the natural phosphodiesterase 2–5A pp5’A(2’ps5’A)₂ (compound 1) was 7 μM, or 17-fold higher. Despite observations that mouse RNase L requires a 5’–diphosphate on 2–5A, the monophosphorylated, PS analogues efficiently activated both mouse and human RNase L (Table 1; Ref. 26). Therefore, these compounds are predicted to be useful in rodent models of prostate cancer. The other advantages of these mixed isomer PS derivatives are a relatively simple mode of preparation compared with stereoisomers, high yields, and a straightforward method of purification.

Apoptosis of Prostate Cancer Cell Lines by 2–5A Activation of RNase L. Results show that 2–5A activation of RNase L leads to RNA damage-mediated apoptosis in the metastatic prostate tumor cell lines DU145, PC3, and, to a lesser extent, LNCaP. Whereas PC3 and DU145 cells are homozygous for fully active forms of RNase L, the LNCaP cells have a deletion/framenutation in one RNASEL allele. LNCaP cells express normal levels of RNase L as the result of allelic compensation. Nevertheless, LNCaP cells were less sensitive to 2–5A treatments than the other two cell lines. For instance, 2–5A produced 39, 27, and 19% RNA breakdown in the DU145, PC3, and LNCaP cells, respectively (Fig. 3). Three days of 2–5A treatment reduced cell viability by 98% and 95% in the DU145 and PC3 cells, respectively, whereas about half of the LNCaP cells survived under identical treatments (Fig. 5). PARP cleavage in response to 2–5A was also greater in the DU145 and PC3 cells than in the LNCaP cells (Fig. 4A). 2–5A activation of RNase L has been shown to lead to release of cytochrome c from mitochondria and to caspase 3-dependent apoptosis (19). In these studies, caspase 3 cleavage in response to 2–5A treatment occurred to a greater extent in DU145 cells than in the PC3 and LNCaP cells (Fig. 4B). Therefore, PARP cleavage was a better indicator of cell death than caspase 3 cleavage. These results suggest that in the LNCaP cells a truncated RNase L produced from the mutant allele may act as an inhibitor of RNase L or that the LNCaP are less efficient in 2–5A uptake than the DU145 and PC3 cells. However, transfection of a fluorescein-tagged 2–5A into the three cell
lines did not show a large difference in transfection efficiencies as measured by cytofluorimetry (data not shown). Therefore, it remains possible that the LNCaP cells produce a RNase L-truncated polypeptide that acts as dominant negative.

The R462Q Variant of RNase L Has a Reduced Capacity to Induce Apoptosis in Response to 2-5A. Three inactivating mutations and an additional nine missense variants in RNASEL have been observed collectively in prostate cancer cases and/or in controls (Fig. 1) (1, 11, 13–16). All three inactivating mutations (M1I, E265X, and Δ157) and six missense variants (G59S, I97L, I220V, V247M, G296V, and S322F) map to the N-terminal half of RNase L that binds 2-5A, whereas the remaining three missense variants (R462Q, Y529C, and D541E) are in the protein kinase-like region. No mutations or variants have been observed in the RNase domain (Fig. 1). Among the missense variants that have been examined in prostate cancer cases (S406F, D541E, I97L, and R462Q), only the R462Q variant has been shown to be associated with prostate cancer risk or aggressiveness (11, 14, 15). To determine the effect of the various missense mutations on enzyme activity, the wild-type and mutant forms of RNase L were compared after expression in mouse JM03 cells, isolated from a spontaneous rhabdomyosarcoma from RNase L+/- p53-/- double gene knockout mice. The R462Q variant showed the lowest levels of enzyme activity, approximately one-third of wild-type RNase L. The Y529C variant was reduced by ~35%, whereas the other variants showed similar activity to the wild-type enzyme. These findings are consistent with genetic evidence implicating, thus far, only R462Q in prostate cancer risk.

A deficiency in RNase L R462Q was investigated further in this study using recombinant purified protein. Enzyme kinetics indicated a 3-fold reduction in catalytic rate compared with wild-type enzyme, consistent with our previous results (15). Whereas 2-5A binding activity was unaffected, the defect was related to a decreased capacity of the enzyme to dimerization into its active form. Previously, we found that another amino acid residue in the protein kinase-like domain of RNase L, K392, was required for dimerization (27). Our current results support a role for the protein kinase homology region in enzyme dimerization. The R462Q mutation reduced the ability of RNase L to cause apoptosis in response to activation by 2-5A as measured by cleavage of PARP and caspase 3 (Fig. 10). Therefore, the association of the R462Q variant with prostate cancer risk correlates with a deficiency in apoptosis. These findings lend additional support to the notion that the tumor suppressor function of RNase L is related to its apoptotic activity.

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