

MATERIALS AND METHODS

Materials. Wild-type bovine pancreatic ribonuclease (RNase A), *Escherichia coli* β -galactosidase (β -gal), and bafilomycin A1 were from Sigma–Aldrich (St. Louis, MO). 5-Chloromethylfluorescein di- β -D-galactopyranoside (CMFDG) was from Invitrogen (Carlsbad, CA).² Quantum™ FITC MESF beads were from Bangs Laboratories (Fishers, IN). All other chemicals and reagents were of commercial reagent grade or better, and were used without further purification.

Small hairpin RNA construct named “sh#1”, which targets human *GLB1* mRNA (M34423), was a generous gift from D. C. DiMaio (Yale University).¹ The anti-GLB1 antibody used in immunoblotting was from Abcam (Cambridge, MA).

The *lacZ* gene, which encodes β -gal, was inserted into plasmid pCMMP-MCS-IRES-mRFP, which was a generous gift from B. Sugden (University of Wisconsin–Madison) and is available as plasmid 36972 from Addgene (Cambridge, MA).²

Mammalian Cell Culture. HeLa cells and cell culture medium and supplements were from the American Type Culture Collection (Manassas, VA) and grown in modified Eagle’s medium containing fetal bovine serum (10% v/v), penicillin (100 units/mL), and streptomycin (100 μ g/mL). *GLB1* knockdown HeLa cells were created by transfecting HeLa cells with the sh#1 construct and continuous selection with puromycin (10 μ g/mL) from Sigma–Aldrich (St. Louis, MO). HeLa cells were grown at 37°C in a humidified incubator containing CO₂(g) (5% v/v).

Instrumentation. Molecular mass was determined by MALDI–TOF mass spectrometry using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA) with sinapinic acid as a matrix in the campus Biophysics Instrumentation Facility. Fluorescence spectroscopy was performed with a QM-1 fluorescence spectrometer equipped with sample stirring from Photon Technologies (South Brunswick, NJ). Flow cytometry data were collected in the University of Wisconsin Paul P. Carbone Comprehensive Cancer Center with a FACSARIA SORP Sorter equipped with UV, 405-nm, 488-nm, 532-nm, and 640-nm solid-state laser (Becton Dickinson, Franklin Lakes, NJ). Microscopy images were obtained with a Nikon C1 laser scanning confocal microscope with a 60 \times oil immersion objective with NA 1.4.

Production and Purification of A19C RNase A. The A19C variant of RNase A was produced and purified as described previously.^{3,4} A19C RNase A contains a free cysteine residue for site-specific conjugation. During protein purification, the free sulfhydryl group was protected as a mixed disulfide by reaction with 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB). Following purification, solutions of A19C RNase A were dialyzed against PBS and filtered (0.2- μ m pore size) prior to use. A19C RNase A concentrations were determined by UV spectroscopy using an extinction coefficient of $\epsilon_{278} = 0.72$ (mg/mL)⁻¹cm⁻¹.⁵

Fluorogenic Labeling of A19C RNase A. Immediately prior to fluorophore attachment, NTB-protected A19C RNase A was deprotected with a 4-fold molar excess of dithiothreitol and desalted by chromatography on a PD-10 desalting column from GE Biosciences (Piscataway, NJ). Deprotected A19C RNase A was reacted with 5-iodoacetamidofluorescein as described previously to generate a simple (that is, unmasked) fluorescein–RNase A conjugate,⁶ or with a 2-fold molar excess of CMFDG for 6 h at ambient temperature (~23°C) followed by 18 h at 4°C. The ensuing FDG–RNase A conjugate was purified by chromatography using a HiTrap SP HP column from GE Biosciences. The molecular mass of RNase A and its conjugates was confirmed by MALDI–TOF mass spectrometry. Conjugate concentrations were determined by using a bicinchoninic acid (BCA) assay kit from Pierce (Rockford, IL) with wild-type RNase A as a standard.

Fluorescence Spectroscopy. The fluorescence released from FDG–RNase A was recorded at ambient temperature (25°C) with excitation at $\lambda_{\text{ex}} = 495$ nm and emission at $\lambda_{\text{em}} = 519$ nm. For pH-sensitivity studies, DFG–RNase A was incubated in 50 mM sodium phosphate buffer (pH 4.0) and PBS buffer (pH 7.2) at 37°C. At known times, the fluorescence of aliquots was measured in PBS buffer (pH 7.2). DFG–RNase A was assessed as a substrate by incubating the conjugate (0.5 μM) with excess β -gal.

Flow Cytometry. The internalization of fluorescein–RNase A and DFG–RNase A was monitored in live HeLa cells. HeLa cells from near confluent flasks were plated in 6-well plates at 1.2×10^5 cells/mL/well 18–24 h prior to transfection. The next day, cells were transfected with the *lacZ*–IRES–*mRFP* vector. After 24 h, fluorescein–RNase A or DFG–RNase A (to 5 μM) was added in each well. At known times, aliquots of HeLa cells were washed extensively with ice-cold PBS, and treated with trypsin/EDTA (0.05% w/v) for several minutes. Complete medium containing FBS (10% v/v) was added, and the aliquots were kept at 0°C until analyzed by flow cytometry.

The fluorescence of fluorescein–RNase A and unmasked DFG–RNase A was detected through a 525/50-nm band-pass filter. mRFP fluorescence was detected through a 610/20-nm band-pass filter. Cell viability was determined by staining with DAPI, which was detected through a 450/40-nm band-pass filter. The mean channel fluorescence intensity of 10,000 viable cells was determined for each sample with FlowJo software.

Fluorescence Microscopy. HeLa cells were treated with DFG–RNase A as for flow cytometry analysis. Viable cells were imaged on a Nikon Eclipse TE2000-U laser scanning confocal microscope equipped with a Zeiss AxioCam digital camera. Excitation at 408 nm was provided by a blue-diode laser, and emission light was passed through a filter centered at 450 nm with a 35-nm band-pass. Excitation at 488 nm was provided by an argon-ion laser, and emission light was passed through a filter centered at 515 nm with a 40-nm band-pass. Excitation at 543 nm was provided by a HeNe laser, and emission light was passed through a filter centered at 605 nm with a 75-nm band-pass.

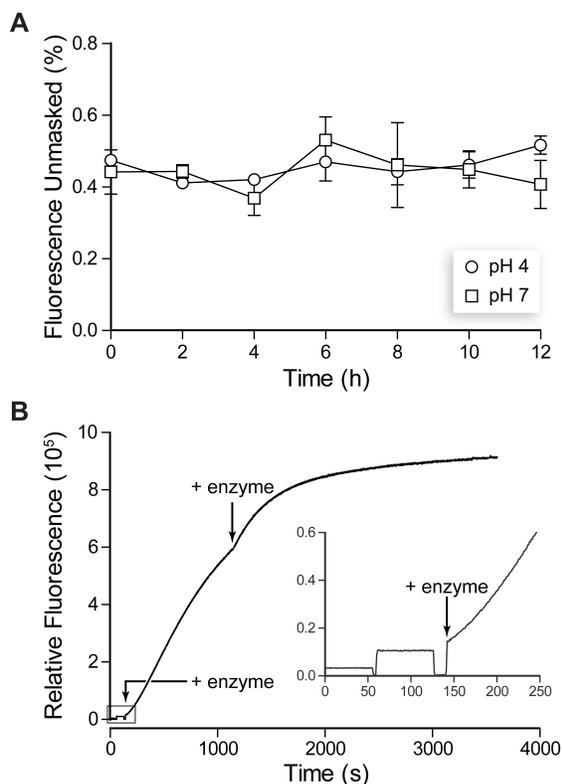


Fig. S1. FDG-RNase A is stable and a substrate for β -galactosidase. (A) The hydrolytic stability of FDG-RNase A was determined by incubation at 37°C in PBS buffer (pH 7.2) or 50 mM sodium phosphate buffer (pH 4.0). At each time point, the fluorescence of FDG-RNase A was measured in PBS buffer (pH 7.2). (B) The enzyme-catalyzed hydrolysis of FDG-RNase A (0.5 μ M) was determined by its incubation with β -gal and monitoring fluorescence ($\lambda_{\text{ex}} = 495$ nm; $\lambda_{\text{em}} = 519$ nm).

Notes and references

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