Inhibition of HIV-1 Production and Selective Degradation of Viral RNA by an Amphibian Ribonuclease*

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Ribonucleases appear to have physiologic roles in host defense against cancer, viruses, and other parasites. Previously it was shown that select ribonucleases added to cells concurrently with virions blocked human immunodeficiency virus, type I (HIV-1) infection of H9 cells. We now report that a ribonuclease homologous to RNase A, named onconase, inhibits virus replication in chronically HIV-1-infected human cells without killing the virally infected cell. Examining the mechanism of this inhibition shows that onconase enters the infected cells and degrades HIV-1 RNA without degrading ribosomal RNA or the three different cellular messenger RNAs analyzed. The homologous human pancreatic RNase lacks anti-viral activity. Comparing recombinant forms of onconase and a onconase-human RNase chimera shows that the N-terminal 9 amino acids and the pyroglutamyl residue of onconase are required for full anti-viral activity. Thus extracellular ribonucleases can enter cells, metabolize select RNAs, and inhibit HIV virion production within viable replicating cells.

Ribonucleases may play anti-viral roles in animals and plants (1, 2). The antiviral activity of interferon can result from activation of a 2'-5'-oligoadenylate-dependent RNase that may specifically degrade viral RNA (3), and in certain plants, viral infection results in RNase induction that may block viral replication (4, 5). Recently an amphibian ribonuclease has been found to express antiviral activity (6). The protein, called onconase or P-30, is isolated from oocytes and/or early embryos of Ranapipiens and is 30% homologoustobovineRNaseA. Main active site residues and three of the four disulfide bonds characteristic of pancreatic RNases are conserved in onconase. However, onconase appears to have a unique catalytic mechanism due to an N-terminal pyroglutamate residue (Glu 4) that folds into the active site (7,8). Onconase was discovered based upon anti-cancer activity and is now in phase III clinical trials for therapy of solid tumors (9, 10).

Previously we found that onconase causes dramatic inhibition of HIV-1 infection of H9 cells at concentrations nontoxic to uninfected H9 cells (6). The onconase was added to normal cells prior to the HIV virions and was thought to enter the cytosol during viral penetration and selectively kill virally infected H9 cells, thereby blocking virus production by the mechanism proposed for a series of plant anti-viral proteins (11, 12). The current study was aimed to explore the mechanism of onconase action and its effect on H9 and U937 cells persistently infected with HIV-1 isolates.

EXPERIMENTAL PROCEDURES

RNases—Onconase was purified from frog eggs (9). Recombinant RNases were constructed, expressed, and purified as reported (8).

Cells and p24 Assays—CD4-positive H9 lymphocyte cells persistently infected with HIV-1 IIIIB strain and the MN isolate of HIV-1 were obtained from Dr. Robert Gallo (National Cancer Institute, NIH, Bethesda, MD). H9 cells were also persistently infected with the MN strain of HIV-1, and U937 cells were persistently infected with either the IIIB strain or the MN strain of HIV-1. These cells were grown in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and 5 μg/ml gentamycin. Prior to use in onconase inhibition studies, persistently infected H9 cells were washed extensively to reduce levels of free virus and resuspended at 2 × 10⁵ cells/ml for use in experimental protocols. Either 1 × 10⁻⁸ or 5 × 10⁻⁸ m onconase was added to washed cells, and cultures were sampled daily over a 5-day period. After the incubation, cells and medium were harvested by centrifuging the culture at 400 × g for 10 min. The supernatant medium was filtered through a 0.45-mm pore size membrane, aliquoted, quickly frozen, and stored at −70°C. Cell pellets were processed to analyze RNA, and the supernatant was used to determine p24 antigen levels. The p24 antigen concentrations were determined by quantitative HIV-1 p24 antigen capture enzyme immunoassay performed according to kit specifications (Coulter).

Cell Growth and Viability Assay—Growth of normal H9 and U937 cells was determined in the presence of onconase up to 3 days. Each initial 50-mL culture had 2 × 10⁶ cells/mL, and viability was determined by trypan blue exclusion.

RNA Extraction and Northern Blot Analysis—1 × 10⁶ H9 cells (cultured in 75-cm² flasks) were incubated with 1 × 10⁻⁸ m and 5 × 10⁻⁸ m onconase for 5 days. Each day one flask of cells was processed as follows to analyze the RNA. Total RNA was extracted using RNAzol according to the protocol supplied by Tel-Test Inc. (Friendswood, TX). Northern blot analysis was carried out according to protocols described in Sambrook et al. (13). Briefly, ~1.5 μg of total RNA from each set was denatured for 15 min at 55°C in 20 μl of 20 mM morpholinopropane sulfonic acid sodium salt and 0.7 M NaOH (pH 7.0) containing 5 mM sodium acetate and 1 mM EDTA (pH 8.0), 50% formamide, and 6.5% formaldehyde and 2 μl of RNA sample buffer (50% glycerol, 1 mM EDTA (pH 8.0), 0.25% bromophen blue, and 0.25% xylene cyanol) and a small amount of ethidium bromide. These samples were electrophoresed on a denaturing 14% agarose gel containing 6.7% formaldehyde and 1 X MOPS buffer (20 mM morpholinopropane sulfonic acid NaOH (pH 7.0) containing 5 mM sodium acetate and 1 mM EDTA (pH 8.0)) at 100 V for 3–4 h. The gels were equilibrated in 20 × SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 45 min before overnight capillary blotting onto Nytran Plus membranes (Schleicher & Schuell) in 10 × SSC. RNA was fixed on to filters by UV cross-linking (UV Stratalinker, Stratagene, La Jolla, CA). Pre-hybridization was carried out at 42°C for 4 h in hybridization buffer (0.2% polyvinylpyrrolidone, M, 40,000), 0.2% ficol (M, 400,000), 0.2% bovine serum albumin, 0.05 M Tris-HCl, pH 7.5, 1 mM sodium chloride, 0.1% sodium pyrophosphate, 1% SDS, 10% dextran sulfate (M,
500,000, and denatured salmon sperm DNA (0.1 mg/ml)). Following prehybridization, hybridization was carried out for 20 h at 42°C using the 32P-Nick translated ([α-32P]dCTP, DuPont NEN) HIV-1-specific; 8-kb DNA probe (AvaI digested 8,088-base pair DNA fragment of pNL4-3;) (14). The blot was washed twice in 2× SSC/1% SDS at room temperature for 15 min each and once in 0.2× SSC/1% SDS at 65°C for 30 min. Autoradiography was carried out with intensifying screens at 270°C and developed by X-Omat (Kodak).

RESULTS

Onconase Blocks p24 Production in Cells Persistently Infected with HIV-1—The addition of onconase to cells blocked p24 production from chronically HIV-1IIIB-infected H9 cells. Onconase at 1×10^−8 M completely blocked p24 antigen production for 2 days, and 5×10^−8 M onconase completely blocked p24 antigen production for at least 5 days (Fig. 1A). In H9 cells chronically infected with another strain of HIV-1, MN (15), onconase at the same concentration inhibited HIV-1 production 60–75% over a 5-day period (Fig. 1B). We also studied U937 cells persistently infected with these two HIV-1 strains (IIIB and MN) and found that onconase also inhibited p24 antigen production of both strains (Fig. 2, A and B). The mammalian cells were persistently infected with HIV, indicating that steps in the HIV-1 life cycle subsequent to genomic integration are susceptible to ribonuclease intervention.

Onconase Cytotoxicity to H9 and U937 Cells—Although onconase was not cytotoxic to H9 cells below 1×10^−7 M (6), we examined whether onconase in the 10^−8 M concentration range slowed the rate of cell division in H9 cells (Fig. 3A) and U937 cells (Fig. 3B). At 5×10^−8 M, onconase was not cytotoxic to cells and only slightly inhibited the H9 cell growth rate and did not affect U937 cell growth rate (Fig. 3, A, B, and C). Therefore, onconase directly inhibits HIV-1 production within viable and dividing cells. These results disprove our previous “Trojan horse” model for onconase antiviral activity (6) and beg the question of mechanism.

Onconase Degradation of Viral RNA—To investigate the molecular basis of RNase anti-viral action leading to the reduction in HIV p24 antigen, the levels of HIV-1 RNA at various times during exposure to onconase were analyzed. Northern blot analysis of the total RNA from uninfected H9 cells and chronically HIV-1IIIB-infected H9 cells treated with different doses of onconase showed that onconase caused a large decrease in the levels of all the HIV-1 RNA transcripts (Fig. 4A). The higher molecular weight HIV-1 transcripts were most susceptible to onconase (Table I). Onconase at 20–100-fold higher doses (1×10^−6 to 1×10^−5 M) can enter the cell cytosol in great enough amounts to degrade cellular RNA (16). However, experiments such as Fig. 4 provide the first evidence that an RNase at one-hundredth the cytotoxic concentration can actually enter cells and degrade viral RNA species. Consistent with this model are recent results showing that disruption of intracellular traffic through the Golgi apparatus can greatly increase the delivery of onconase to the cytosol (17, 18). The most dramatic decrease in HIV-1 RNA levels occurred with 5×10^−8 M onconase and lasted up to 4 days. After 4 days of exposure to the initial onconase dose, HIV-1 RNA levels began to increase (Fig. 4A). This increase in HIV-1 RNA level after 4 days of inhibition precedes the increase in p24 antigen concentrations seen in
Onconase treatment at $1 \times 10^{-8} \text{ M}$ resulted in a similar inhibition of HIV-1 RNA levels, although the inhibition at this lower dose of onconase was shorter in duration than for the $5 \times 10^{-8} \text{ M}$ onconase-treated cells. This may reflect the half-life of onconase within cultured cells. When cells were treated with $1 \times 10^{-8} \text{ M}$ onconase, the eventual reappearance of the HIV-1 RNA after 3 days corresponded with renewed p24 antigen production (Figs. 1A and 4A). These results also show that the ribonuclease is not lethal to the cells because they re-express HIV-1 RNA and p24 antigen with increased time after ribonuclease exposure.

Because of the eventual resynthesis of HIV-1 RNA, we added a second dose of onconase at $1 \times 10^{-8} \text{ M}$, 2 days after an initial onconase treatment at $1 \times 10^{-8} \text{ M}$ on day zero. The second dose of onconase caused a significant further decrease in p24 antigen production and in cell division rate. However, the inhibition of p24 antigen by the second treatment was less than that of the first exposure to onconase (data not shown). Comparing the RNA from H9 cells treated on day 0 only and those treated at day 0 and day 2 with $1 \times 10^{-8} \text{ M}$ onconase revealed that the increase in HIV-1 RNA levels seen 4 and 5 days after a single inoculum of onconase (Fig. 4A) was blocked by the second addition of onconase (Fig. 5A). Thus repeated application of ribonucleases has repeated antiviral effects.

Onconase Does Not Degrade Ribosomal RNA and mRNAs—Examining the ribosomal RNA from the same H9 cell samples where viral RNA was destroyed showed no effect of onconase on rRNA (Fig. 4B). We also examined the levels of messenger RNAs encoding human actin, glyceraldehyde-3-phosphate dehydrogenase, and the transferrin receptor in $5 \times 10^{-8} \text{ M}$ onconase-treated and control H9 cells (Fig. 6). No decrease in mRNA levels was found in three out of the three mRNAs examined. Thus onconase expressed a surprising selectivity for HIV-1 RNA species within cells, a finding consistent with its low cytotoxicity and minor effect on cell growth rate. However, tRNA may also be highly susceptible to degradation by onconase and certain homologous RNases (19, 20). As onconase is not likely to express sequence specificity for HIV RNA, perhaps proteins complexed with RNA protect endogenous cellular RNAs from onconase to a greater extent than the HIV-1 RNAs.

Infectivity of HIV-1 Virions Produced from Onconase-treated Cells—Although onconase blocks virus production by a new and unknown mechanism, the HIV-1 DNA integrated into the mammalian genome eventually begins virion production at some time following the ribonuclease exposure (Figs. 1 and 2).
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The autoradiographic film from Fig. 4A was scanned and analyzed by the NIH Image 1.55 program. The peak area for different HIV-1RNA species from the Day 0 sample lanes was used at 100%. The autoradiographic film from Fig. 4A was scanned and analyzed by the NIH Image 1.55 program. The peak area for different HIV-1RNA species from the Day 0 sample lanes was used at 100%.

Quantitation of different HIV-1RNA species

| HIV-1 RNA species | 5 × 10^{-8} M Onconase | 1 × 10^{-8} M Onconase |
|-------------------|------------------------|------------------------|
|                   | Day 1      | Day 2      | Day 3      | Day 4      | Day 5      | Day 1      | Day 2      | Day 3      | Day 4      | Day 5      |
| ~8 kb             | 4          | 5          | 6          | 25         | 27         | 14         | 3          | 14         | 36         | 119        |
| ~4 kb             | 33         | 47         | 37         | 60         | 78         | 64         | 44         | 61         | 100        | 118        |
| ~2 kb             | 37         | 27         | 35         | 23         | 47         | 59         | 75         | 35         | 77         | 92         |

Thus it will be important to examine effects of ribonucleases on viral penetration and integration because these steps may also be uniquely susceptible to ribonucleases and allow a permanent elimination of virus production.

We compared HIV-1 infectivity titers on virus harvested from onconase-treated H9 cells and untreated controls. Titers of virus from onconase-treated and untreated cells standardized for the same amount of p24 antigen showed no relative difference in infectivity (data not shown). Thus the virus eventually produced by onconase-treated cells was not defective. The RNA genome and associated primer tRNAs (21) packaged into virus particles appear to function correctly when packaged from onconase-treated cells. In the future it may be possible to enhance onconase antiviral activity by engineering amino acid sequences into onconase that package it into HIV virions (22) or that target it selectively to HIV-infected cells (23, 24).

Structural Determinants in Antiviral Activity—Previously it was found that human eosinophil-derived neurotoxin and bovine pancreatic RNase A, both members of the ribonuclease family homologous to onconase in sequence, lacked antiviral activity (6). We constructed several recombinant forms of onconase (8) and used these variants to probe the structural differences between human pancreatic RNase and onconase that generate antiviral activity (Fig. 7A). When onconase was expressed with an N-terminal methionine instead of the pyroglutamyl residue found in the native frog protein, ribonuclease activity was decreased 80−90% (8). We compared the antiviral activity of native onconase, recombinant onconase expressed with an N-terminal methionine residue, and recombinant onconase treated with cyanogen bromide to reconstitute the N-terminal pyroglutamyl residue. Fig. 7B shows that whereas native onconase and the recombinant pyroglutamyl form of onconase expressed potent antiviral activity, the N-terminal methionine form of onconase completely lacked activity. Thus antiviral activity correlates with the degree of ribonuclease activity as the two pyroglutamyl forms of onconase are more active in both respects than the N-terminal methionine form.

DISCUSSION

Ribonucleolytic antiviral activity is reminiscent of the pathway by which interferon inhibits replication of a number of viruses including HIV-1 (25, 26). Interferon induces a 2′-5′ oligoadenylate-dependent ribonuclease (27) as well as 2′-5′ oligoadenylate synthetases that when triggered by virus produce the allostERIC activator of the 2′-5′ oligoadenylate-dependent RNase. The activated 2′-5′ A-dependent RNase cleaves HIV viral RNA as well as ribosomal RNA and can inhibit HIV replication in vitro (28), and interferon can ameliorate the
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clinical symptoms of HIV-1, at least temporarily (29). Like onconase, interferon induction of the 2-5A-dependent RNase also slows cell division rates (30). Another important feature shared by onconase and interferon is that they both inhibit viral production even when administered after cells are infected with HIV-1 (31). This contrasts with reverse transcriptase inhibitors such as azidothymidine that block new infection cycles but do not inhibit viral production from chronically infected cells. This is a particularly important attribute shared by interferon and onconase that may have therapeutic importance. It has already been noted that combinations of agents such as azidothymidine and interferon, which act early and late in the infection cycle, respectively, act synergistically (32, 33). Consistent with these results and the suggestion that onconase and interferon work at similar steps in viral infection, we find that combinations of an interferon and onconase do not act synergistically or additively.2 Given the high viral production and turnover during AIDS that continues for days even after inhibition of new viral infection cycles with noncompetitive reverse transcriptase inhibitors and protease inhibitors (34, 35), onconase administered simultaneously with protease and/or reverse transcriptase inhibitors may block virus production from persistently infected cells and may decrease the probability of new variant HIV-1 particles arising that are resistant to the antiviral agents. The time course of viral production and cell turnover coincide well with the safe regimens of onconase currently in clinical trials for cancer (10).

These results also suggest a new physiologic step in the diverse activities of extracellular ribonucleases. Onconase is homologous to a family of proteins, many of which exist in mammals, that has well understood biochemical properties and evolutionary relationships but poorly understood physiological roles. The pancreatic RNase family members have a variety of biologic effects on cells and tissues that depend upon RNase enzyme activity (1, 2, 36, 37). However, all members of this superfamily have a signal peptide that directs their secretion or sequestration within vesicles (38), segregating them from any known RNA. How extracytoplasmic RNases may alter cells via RNA substrates has been difficult to explain (39). Our results, showing that one member of the pancreatic ribonuclease family can enter cells and alter specific RNA transcript levels without blocking cell growth, suggest that other members of this family may have related activities that could contribute to their diverse biologic properties. Furthermore, these results suggest that certain members of the pancreatic ribonuclease superfamily may have physiologic roles as anti-viral proteins.

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