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CHARACTERIZATION OF THE MECHANISM OF CELLULAR AND CELL FREE PROTEIN SYNTHESIS INHIBITION BY AN ANTI-TUMOR RIBONUCLEASE *,+

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SUMMARY: Onconase, a protein with anti-tumor activity, causes potent inhibition of protein synthesis in the rabbit reticulocyte lysate ($IC_{50}10^{-11}$M) and when microinjected into *Xenopus* oocytes ($IC_{50}10^{-10}$M). Onconase is a member of the RNase A superfamily; however, unlike RNase A, the mechanism of protein synthesis inhibition does not involve apparent degradation of lysate or cellular ribosomal RNAs. Rather, reticulocyte and oocyte tRNA is hydrolyzed after Onconase treatment. Furthermore, re-addition of tRNA to Onconase pretreated lysates or oocytes restores the translational capacity of the system. Taken together these results suggest that Onconase causes potent protein synthesis inhibition by a mechanism involving inactivation of cellular tRNA.

The primary sequence of Onconase is highly homologous to that of pancreatic ribonuclease A (RNase A) (1) and it exhibits RNase activity. Onconase was isolated from extracts of *Rana pipiens* oocytes and early embryos (1) based upon an anti-proliferative/ cytotoxic effect toward cancer cells (2,3). Onconase displays anti-cancer therapeutic activity in animal models (4). A Phase I human clinical trial in patients with a variety of solid tumors (5) as well as a Phase I/II clinical trial have recently been completed. We now report the effects of Onconase on the translational capacity of the rabbit reticulocyte lysate and *Xenopus* oocytes. Onconase is a potent inhibitor of both cell free and cellular protein synthesis by a mechanism involving tRNA inactivation.

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Experimental Procedures

Materials: Onconase (previously named P-30 Protein) is a registered trade mark of Alfacell Corporation (Bloomfield, NJ). Onconase, purified as described (1) and goat-anti-Onconase antibody was provided as lyophilized protein and dissolved in phosphate buffered saline (PBS). *Xenopus laevis* was purchased from *Xenopus*-1. Pre-poured polyacrylamide and TBE gels were from Novex. Bovine pancreatic RNase A was obtained from Calbiochem; human placental ribonuclease inhibitor (RNasin), Brome Mosaic Viral mRNA and rabbit reticulocyte lysate translation system from Promega Biotech, tRNA (calf liver) and rabbit globin mRNA were from Gibco-BRL. Yeast RNA was from Sigma. L-[^{35}S]-methionine (1,134 Ci/mmol) was from DuPont-New England Nuclear.

In Vitro Translation Assays: The *in vitro* translation assay was performed as previously described (6). The amount of protein synthesis was determined by the incorporation of [^{35}S]-methionine into products precipitable by 10% trichloroacetic acid (TCA) following the protocol recommended by Promega and by autoradiography of the proteins separated by polyacrylamide gel electrophoresis on 4-20% gradient gels.

Oocyte Microinjection and Protein Synthesis Determination: 46 nl samples were microinjected into the vegetal pole of stage VI oocytes as described (7). For each protein synthesis determination, duplicate wells containing three injected oocytes were labeled with [^{35}S]-methionine (0.2 mCi/ml) in oocyte Ringer (OR) medium for 20 hr at 18°C. After labeling, the medium was removed and the oocytes were rinsed with an excess of OR, homogenized and analyzed for incorporation of [^{35}S]-methionine into proteins as described (8). Calculation of RNase molar concentrations were accomplished using an approximate oocyte volume of 500 nl as described previously (9).

Characterization of Reticulocyte and Oocyte RNA: RNA from control or nuclease treated lysates or oocytes was prepared as described (6). Electrophoretic analysis was accomplished by formaldehyde/agarose gels or 20% TBE gels.

Results: Onconase is a potent inhibitor of protein synthesis. The IC_{50} of Onconase to inhibit protein synthesis in the rabbit reticulocyte lysate and *Xenopus* oocytes was approximately 10^{-11} M and 10^{-10} M, respectively. RNA was isolated from RNase A or Onconase-treated lysates and examined by formaldehyde agarose gel electrophoresis. Although Onconase is a member of the RNase A superfamily, extensive degradation of the major ribosomal RNAs was not discernible from the Onconase treated lysates at concentrations of Onconase that markedly inhibit protein synthesis (Fig. 1A & C). Conversely, as protein synthesis was progressively inhibited by increasing concentrations of RNase A, extensive degradation of rRNA was observed (Fig. 1B & D). Effects on tRNA were examined by analyzing equal amounts of RNA from these experiments by electrophoresis on 20% TBE gels (Fig. 1 E & F). Onconase begins to degrade tRNA at 0.14 nM or at a 1000-fold lower concentration than that required for gross degradation of rRNA. The tRNA appears to be progressively degraded with increasing Onconase concentrations (Fig. 1, E). Conversely, RNase A only begins to degrade tRNA at concentrations that extensively degrade the major ribosomal RNAs (Fig 1, F).

The oocyte has been used as a model system to determine the specific effects of toxins (10) and RNases (9,11) in whole cells. Onconase is a potent inhibitor of protein synthesis in

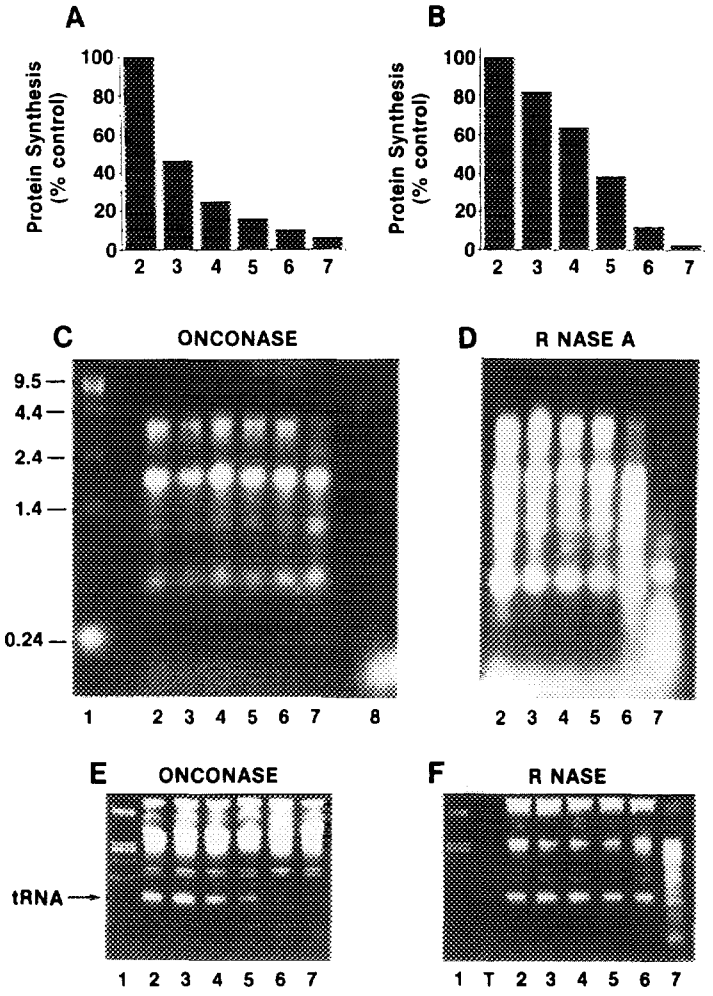

Fig. 1. A & B. Inhibition of protein synthesis in reticulocyte lysates by Onconase or RNase A. (A) Lane 2, Control lysate without nuclease; Lanes 3-7, Onconase 0.014, 0.14, 1.4, 14 and 140 nM, respectively. (B) Lane 2, control without nuclease, lanes 3-7, RNase A same as in A. C & D, Agarose gel electrophoresis of RNA from Onconase or RNase A treated lysates. Reticulocyte RNA was isolated from nuclease treated lysates described in A & B, electrophoresed on a 1.4% formaldehyde/agarose gel and stained with ethidium bromide. (C) Lane 1, RNA ladder from 0.24 to 9.5 Kb, the positions are indicated to the left of the figure, 28S rRNA migrates at approximately 4 Kb and 18S rRNA migrates at approximately 2 Kb; lanes 2-7, same as A, lane 8, tRNA standard; (D) lanes 2-7, same as B. E & F, Electrophoresis of Onconase or RNase A treated reticulocyte RNA described in A&B on a 20% TBE gel. The RNA samples were stained with ethidium bromide. (E) Lane 1, Phi X 174 /Hae III Fragments, 194-1353 bp; Lanes 2-7, same as A. Densometric analysis using Image analysis 4.5 was used to scan the tRNA band for lanes 3-7 and generated the following values: 100, 82, 43, 14 & 0% of control; (F) Lane 1, Phi X 174 /Hae III Fragments; Lane T, tRNA standard; Lanes 2-7 same as B; Densometric analysis values for lanes 3-7: 100, 100, 92, 100, and 0% of control.

intact oocytes (Fig. 2). Total oocyte cellular RNA isolated from control and Onconase treated oocytes was analyzed by agarose gel electrophoresis (Fig.2, bottom). Consistent with the data shown in Fig. 1, there is no obvious degradation of the rRNA with concentrations of Onconase

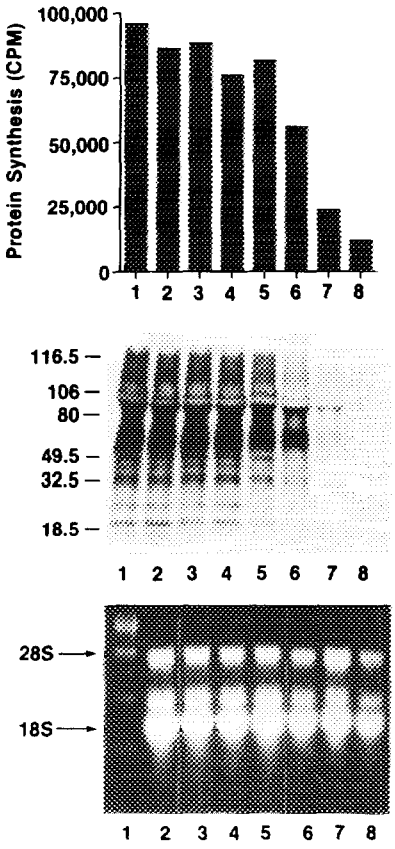

Fig. 2. Microinjected Onconase inhibits protein synthesis in *Xenopus* oocytes without apparent degradation of rRNAs. Top, Onconase was injected and protein synthesis determined as described in Experimental Procedures. Lanes 1-8, uninjected, 0.01% gelatin injected, 0.01% gelatin containing Onconase $10^{-12,-10,-8,-6,-4,-2}$ mg/ml. The final concentration of Onconase in the oocytes injected with 10^{-2} mg/ml was approximately 100 nM. Middle, [^{35}S]-methionine labeled oocyte proteins analyzed on a 4-20% gradient SDS acrylamide gel. Lanes 1-8 are exactly as described in the top panel. Molecular weight markers are indicated to the left of the figure. Bottom. Total oocyte RNA was isolated and analyzed on a 1.4% agarose gel as described in Experimental Procedures. Lane 1, RNA molecular weight markers corresponding to 1.4, 2.4, 4.4, 7.5 and 9.5 Kb, respectively. Lane 2, uninjected control, lane 3,diluent (0.01% gelatin), lanes 4-8, Onconase $10^{-6,-5,-4,-3,-2}$ mg/ml, respectively. Positions of the 28S and 18S rRNAs are indicated by the arrows.

that completely inhibited protein synthesis even 18 hr after the injection of Onconase. Again, this is different from the results observed after microinjection of RNase A. Increasing concentrations of RNase A inhibited oocyte protein synthesis and caused visible destruction of oocyte cellular rRNAs (not shown and 9). Since Onconase did not degrade oocyte rRNA, the effect of Onconase to degrade microinjected exogenous tRNA was examined. Kinase labeled tRNA was prepared, injected and found to be degraded to 78% of control values after 1 hr and 25% of control values after 16 hr.

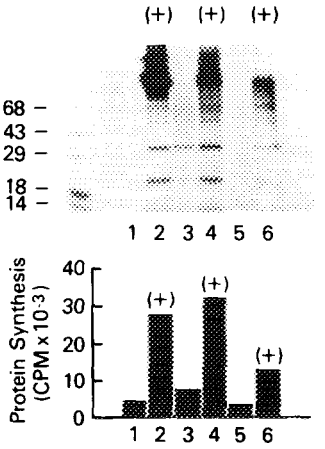

Fig. 3. Re-addition of tRNA to Onconase treated rabbit reticulocyte lysate restores the translational capacity of the lysate. Reticulocyte lysate was treated with Onconase for 15 min at 30°C. The ribonucleolytic activity of Onconase was stopped with anti-Onconase antibody (1.3 μg/50 μl reaction). Translation was initiated with or without the addition of exogenous calf liver tRNA (50 μg/ml final concentration) and continued for an additional 60 min at 30°C. Protein synthesis was determined as described in Experimental Procedures. The reactions were analyzed by SDS-polyacrylamide gel electrophoresis (Top) and by determining TCA precipitable CPM (Bottom). (Lanes 1-6, top and bottom) 1, reaction with all components necessary for translation, except mRNA; 2, reaction with all components necessary for translation plus mRNA; 3 and 5, same as 2 plus Onconase (0.014 or 0.14 nM); 4 and 6, same as 3 and 5 plus tRNA added after the anti-Onconase antibody at 15 min. The molecular weight markers are indicated to the left of the top panel. (+) indicates the re-addition of tRNA.

Rabbit reticulocyte lysate was incubated with Onconase for 15 min followed by the addition of anti-Onconase antibody to block further Onconase activity. *In vitro* translation was initiated with or without exogenous tRNA. Analysis by SDS-polyacrylamide gel electrophoresis (Fig. 3) shows that Onconase decreases the appearance of [^{35}S]-methionine labeled proteins in a concentration dependent manner. This demonstrates that the observed decrease in CPM reflects a decrease in the incorporation of label into newly synthesized proteins (Fig. 3 top, lanes 3 and 5). This is quantitated in Fig. 3 (bottom, lanes 3 and 5). The addition of tRNA reactivated the translational capacity of the system Fig. 3 (lanes 4 and 6). In other experiments we showed that total yeast RNA did not restore protein synthesis after Onconase treatment (not shown).

Similar reconstitution experiments were performed after Onconase pre-treatment of *Xenopus* oocytes. Four hours after injecting oocytes with Onconase, the oocytes were reinjected with tRNA in the presence of Onconase antibody to inhibit further Onconase activity. RNasin was included to inhibit trace contaminants in the Onconase antibody that became problematic with the longer incubations in the oocyte. Protein synthesis was measured 18 hours later.

Onconase inhibition of protein synthesis was restored in the oocytes that had been reinjected with tRNA (Table 1).

Discussion: Analysis of RNA isolated from Onconase treated lysates revealed that Onconase does not cause detectable degradation of the major reticulocyte rRNAs at concentrations that have a marked effect on protein synthesis. In contrast, RNase A causes destruction of reticulocyte rRNA at concentrations that closely correlate with protein synthesis inhibition consistent with previously published results (6,9). Hydrolysis of tRNA corresponds to protein synthesis inhibition by Onconase. Moreover, re-addition of tRNA to Onconase-treated lysates restores protein synthesis but not to RNase A-treated lysates (unpublished data). Therefore, Onconase is a potent inhibitor of protein synthesis by affecting cellular RNAs differently than degradative RNase A.

Regeneration of biological intermediaries and regulatory loops requiring transcriptional signals are not operative in the reticulocyte lysate system. Therefore, we microinjected Onconase into *Xenopus* oocytes and examined effects on protein synthesis in a living cell. Even after extended incubation of Onconase in a dynamic system, we obtained results similar to those in the lysate: *i*, Onconase inhibited protein synthesis without apparent degradation of rRNA while RNase A inhibition of protein synthesis correlated to rRNA degradation; *ii*, tRNA restored the capacity for protein synthesis after Onconase treatment.

Onconase is an RNase that is being evaluated in the clinic as an anit-cancer agent (5) and this is the first study to demonstrate that tRNA may be involved in its mechanism of action.

Table 1: Reversal of Onconase mediated protein synthesis inhibition in *Xenopus* oocytes by tRNA

ADDITIONS	CPM ($x10^3$)	%CONTROL
Uninjected	104	
0.01% gelatin + AR	81	100
+Onconase[1]	47	58
+Onconase+AR	50	62
+Onconase+ARt	95	117

[1]Onconase, 10^{-6} mg/ml injected

Onconase or 0.01% gelatin lacking Onconase was injected and 4 hr later the oocytes were re-injected with gelatin or gelatin containing anti-onconase antibody (A), RNasin (R), with or without tRNA (t). The injections and extraction of labeled proteins were performed as described in Experimental Procedures.

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