

Activation of ribonuclease F by the two isomers (2'-5') oligoadenylate and (3'-5') oligoadenylate

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Received 20 August 1982; revision received 24 September 1982

Ribonuclease F activation *Oligoadenylate isomers, 2'-5' and 3'-5'*
Phosphodiester linkage *Oligoadenylate synthetase 2'-5'* *Interferon*
ATP polymerization

1. INTRODUCTION

Treatment of cells with interferon (IFN) results in the induction of an enzyme, (2'-5')-oligoadenylate synthetase, which in the presence of double-stranded RNA catalyzes the polymerization of ATP into a series of oligonucleotides with the unique linkage of (2'-5')-phosphodiester [1]. These (2'-5')-oligoadenylate molecules activate an endonuclease, RNase F, which is present in both IFN-treated and untreated cells [2-5]. Activation of RNase F has been implicated in both the antiviral and anticellular activities of IFN [6,7]. Several groups have studied the specificity of interaction between (2'-5')-oligoadenylate derivatives and RNase F. Oligomers of three or more adenylate residues, containing di- or tri-phosphate at the 5'-end, were able to bind and activate RNase F with similar efficiencies. Oligomers with monophosphate at the 5'-end bind RNase F, while dephosphorylated molecules could neither bind nor activate it [3,8,9,15].

Here, we extend these studies and examine the role of the 2'-5'-phosphodiester linkage in the activation of the RNase F.

2. MATERIALS AND METHODS

2.1. Preparation of (3'-5')-ppApApA

Polyadenylate was synthesized by enzymatic polymerization of ADP with *Micrococcus lyso-deikticus* polynucleotide phosphorylase [12] and the (3'-5')-polyadenylate product was purified

[11]. Extensive degradation of polyadenylate product with the nucleases P₁ or T₂ produced 5' and 3' AMP, respectively. The polyadenylate (average sedimentation coefficient of 3 S) was taken for partial hydrolysis by P₁ nuclease [13].

Degradation products were resolved according to charge on a DEAE-cellulose column with a salt gradient (0.02-0.65 M ammonium bicarbonate). Oligonucleotides in the range 1-7 residues are well separated on this column. The oligonucleotide fractions were collected and further characterized by high-voltage paper electrophoresis and paper chromatography.

Characterization was carried out before and after treatment with *Escherichia coli* alkaline phosphatase. The fraction containing (3'-5')-pApApA was taken and salt was removed by repeated lyophilization. The yield of trinucleotide was 10% of the input polyadenylate. The purified (3'-5')-pApApA (5000 A₂₆₀) was phosphorylated at the 5'-end with the tri-*n*-butyl-ammonium salt of phosphoric acid as in [14]. Yields of phosphorylation of several preparations were ~20%. The products of this reaction were fractionated on a DEAE-cellulose column with a salt gradient (0.02-0.65 M ammonium bicarbonate). Two main peaks were resolved by this column. The first eluting material was identified as pApApA by paper chromatography and electrophoresis as above. Both peaks were further characterized by chromatography on thin-layer plates of poly(ethyleneimine)-cellulose [15] and high-pressure liquid chromatography (HPLC) (see fig. 1).

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2.2. Preparation of (2'-5')-ppApApA

For the synthesis of (2'-5')-pApApA, we followed the procedure in [10]. In brief, (2'-3')-cyclic AMP was polymerized as in [19] and the polymer obtained was digested with P_1 nuclease under conditions for a complete cleavage of (3'-5')-phosphodiester bonds [10]. Digestion products were resolved on a DEAE-cellulose column and characterized as in [10] and above. Fractions containing (2'-5')-pApApA were collected and chemical phosphorylation of (2'-5')-pApApA was conducted in parallel with (3'-5')-pApApA. Purification and analysis of the two isomers were carried out by the same method.

3. RESULTS

The oligonucleotide (3'-5')-pApApA was synthesized by enzymatic polymerization of ADP with polynucleotide phosphorylase [11,12], followed by partial degradation with P_1 nuclease [13], and purification of trinucleotide containing 5' phosphomonoester. (2'-5')-pApApA was synthesized by chemical polymerization of (2'-3')-cyclic adenosine monophosphate [10,19], followed by cleavage of all (3'-5')-phosphodiester bonds with P_1 nuclease [10]. The two isomers of pApApA were chemically phosphorylated to produce the final products ppApApA. Both isomers were analyzed by chromatography on thin-layer plates of poly(ethyleneimine)-cellulose [15] and by HPLC, before and after digestions with alkaline phosphatase and P_1 nuclease. The two compounds appeared to be homogenous and of the expected structure. The analysis of (3'-5')-ppApApA by HPLC is illustrated in fig. 1, the compound eluted from the column as a single peak (fig. 1A). Digestion of (3'-5')-ppApApA with P_1 nuclease resulted in two products which eluted from the HPLC as expected at the positions of A5'p (4.3 min) and A5'pp (3.6 min) (fig. 1B).

To study the specificity involved in the activation of RNase F, the oligoadenylate derivatives were co-precipitated with calcium phosphate and added to tissue cultured L-cells. This procedure enabled efficient penetration of oligonucleotides into the cytoplasm and inhibition of cell protein synthesis by RNase F activation [6]. Introduction of (3'-5')-ppApApA into cells resulted in the inhibition of protein synthesis; 50% inhibition was ob-

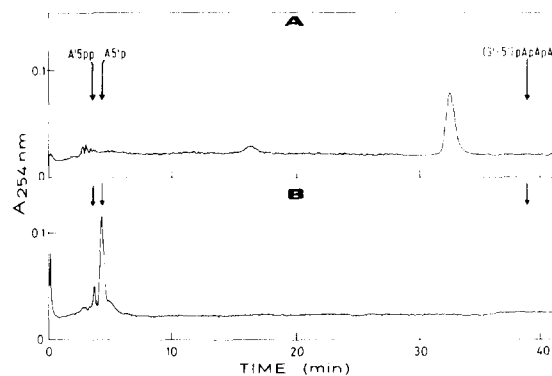


Fig. 1. Analysis of (3'-5')-ppApApA by HPLC. Chromatography was carried out on C-18 column as in [9]. Compounds were eluted with 5 min wash with buffer A (50 mM ammonium phosphate, pH 7.0) at 1 ml/min, a 25 min gradient from buffer A (80%) to 20% methanol: water (1:1); 5 min gradient from buffer A (50%) to 50% methanol: water (1:1) and a 5 min gradient from buffer A (30%) to 70% methanol: water (1:1).

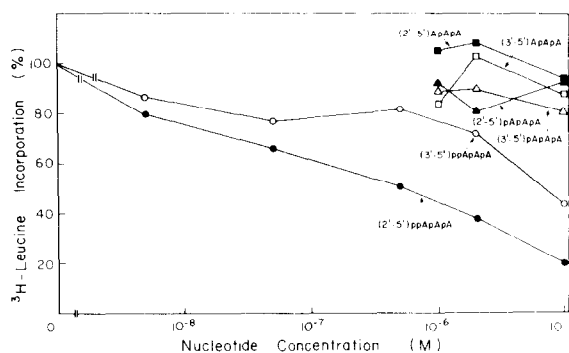


Fig. 2. Activation of RNase F by different derivatives of oligoadenylate. Oligonucleotides were synthesized as in section 2. The calcium phosphate co-precipitation technique was described in [6,16]. L₉₂₉ cells (1×10^5) were seeded in plates with 24 wells (16 mm) in 1 ml RPMI 1640 medium containing 10% calf serum; 48 h after seeding, cultures were incubated with the oligonucleotides co-precipitated with calcium phosphate for 2 h at 37°C [16]. The precipitates were removed and cultures were incubated for another 2 h in 1 ml Eagle's (MEM) medium containing 10% calf serum. Cells were pulse labeled for 1 h with [3 H]leucine (2 μ Ci/0.5 ml MEM medium minus leucine) and protein synthesis was assayed as [16]. The results were determined from the replication in an average of three wells. L-cells without oligonucleotide (100%) incorporated 108000 cpm: (2'-5')-ppApApA (●—●); (3'-5')-ppApApA (○—○); (2'-5')-pApApA (▲—▲); (3'-5')-pApApA (△—△); (2'-5')-ApApA (■—■); (3'-5')-ApApA (□—□).

tained at 4×10^{-6} M (fig. 2). Under the same conditions, the isomer (2'-5')-ppApApA inhibited protein synthesis by 50% at 8×10^{-7} M.

These results indicated that, while (3'-5')-ppApApA could activate RNase F in cells, the activation required 5-10-fold higher concentrations than those of the natural compound (2'-5')-ppApApA. The monophosphate precursors, (2'-5')-pApApA and (3'-5')-pApApA, did not affect protein synthesis after introduction into cells. The dephosphorylated isomers (3'-5')-ApApA and (2'-5')-ApApA, were also inactive in these cells (fig. 2). In the assay system described above, the enzymatically prepared (2'-5')-pppA(pA), (where $n = 2-5$) [20], inhibited protein synthesis by 50% at 5×10^{-7} M (not shown).

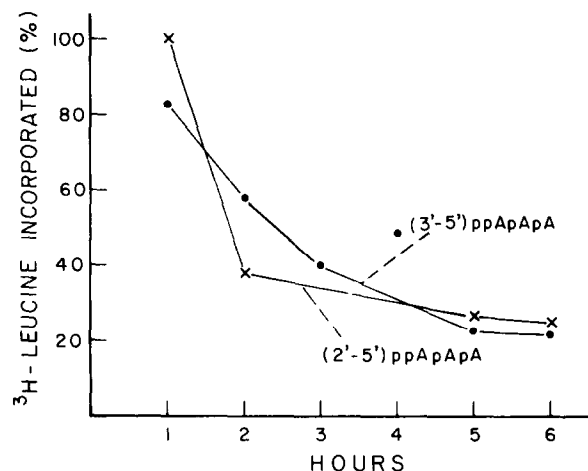


Fig. 3. Kinetics of cellular protein synthesis after treatment with (3'-5')- and (2'-5')-ppApApA. L-cells were treated with (2'-5')-ppApApA 5×10^{-7} M ($\times - \times$), or (3'-5')-ppApApA 5×10^{-6} M ($\bullet - \bullet$), as in fig. 2. After incubation for 2 h with calcium phosphate precipitates, the cultures were washed and MEM medium containing 10% calf serum was added. At various times after the addition of calcium phosphate precipitates, the rate of protein synthesis was assayed by [3 H]leucine incorporation for 15 min. For the 1 h point, cultures were treated with calcium phosphate for 1 h only, washed and pulse labeled for 15 min. Control cultures treated with calcium phosphate but without oligonucleotides were assayed in parallel at each time point, and the percentage of [3 H]leucine incorporated was calculated by taking the incorporation without oligonucleotide at each time point as 100%. In this way, the slight inhibitory effect of the calcium phosphate treatment was taken into account. [3 H]leucine incorporation at zero time before addition of the calcium phosphate precipitate (100%) was 34000 cpm.

To compare the rates of action of the two isomers, we studied the kinetics of protein synthesis inhibited by the two active compounds. When (3'-5')-ppApApA was added to cells at a 10-fold higher concentration than that of (2'-5')-ppApApA, similar kinetics of inhibition were observed (fig. 3). Inhibition of 50% was noticed 2-3 h after addition of the two isomers coprecipitated with calcium phosphate.

Inhibition of cell protein synthesis is an indirect measurement of RNase F activation and RNA degradation. To directly assess activation of RNase F, by (3'-5')-ppApApA and to determine the

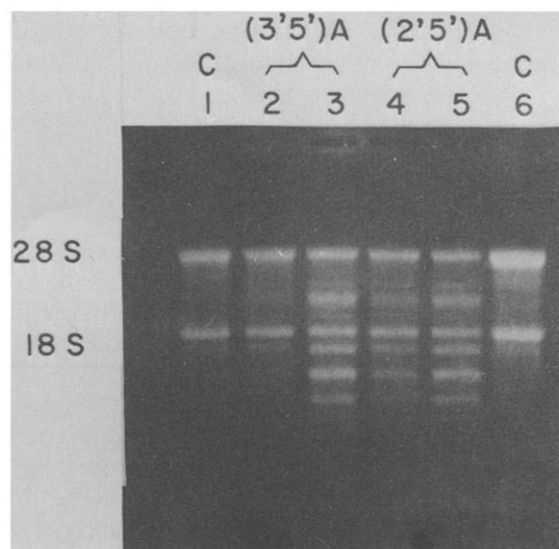


Fig. 4. Ribosomal RNA cleavage in (2'-5')-ppApApA and (3'-5')-ppApApA treated cells. L-cells (20×10^6 in 150 mm plates) were treated with oligonucleotides coprecipitated with calcium phosphate (see fig. 2). After 2 h incubation, the precipitates were removed, cultures were washed, and MEM medium with 10% calf serum was added for 2 h. Cultures were washed with cold phosphate-buffered saline (PBS) and cells lysed by adding buffer A (10 mM Hepes, pH 7.6; 15 mM $Mg(Ac)_2$; 90 mM KCl, 0.5% Nonidet P40). Nuclei were removed by centrifugation for 10 min at $8000 \times g$ and RNA was purified from the cytoplasmic fraction by phenol extraction and ethanol precipitation. RNA, after denaturation with glyoxal, was resolved on agarose gels (1.8%) as in [17] and stained with ethidium bromide: (1) control cells not treated with calcium phosphate; (2,3) cells treated with (3'-5')-ppApApA, 5×10^{-6} and 5×10^{-7} M, respectively; (4,5) cells treated with (2'-5')-ppApApA, 10^{-6} and 10^{-7} M, respectively; (6) control cells treated with calcium phosphate without oligonucleotide.

specificity of its action, we analyzed the degradation of ribosomal RNA which followed introduction of the oligonucleotides into cells.

Activation of RNase F by (2'-5')-pppApApA has been shown to produce a typical pattern of rRNA degradation which could be resolved by electrophoresis on agarose gels [17]. Introduction of (3'-5')-ppApApA and (2'-5')-ppApApA into L-cells activated identical patterns of rRNA cleavage. However, ~10-fold higher concentrations of (3'-5')-ppApApA than of (2'-5')-ppApApA were needed to obtain a similar extent of rRNA degradation (fig. 4). The pattern of rRNA cleavages obtained in this work after addition of (2'-5')- and (3'-5')-ppApApA was very similar, if not identical, to that reported for the activation of RNase F in L-cells by the enzymatically synthesized (2'-5')-pppApApA [17].

4. DISCUSSION

These results indicate that RNase F could be activated by (3'-5')-ppApApA, the isomer of the naturally occurring (2'-5')-ppApApA. Enzymatic polymerization of (2'-5')-phosphodiester bonds in oligonucleotides is unique to the interferon-induced synthetase [1] and the role, if any, of such a rare linkage is not known. Conceivably, RNase F specificity in binding or activation could be restricted to (2'-5')-oligonucleotides. Alternatively, the stability of (2'-5')-oligonucleotides in the cell could be higher than that of (3'-5')-oligonucleotides. A specific phosphodiesterase in L-cells degrades both (3'-5')- and (2'-5')-phosphodiester bonds [18]. Other nucleases, however, are more specific for (3'-5')-oligonucleotides and thus, the latter isomer might be more rapidly degraded in the cells. Additional studies of binding and activation with (3'-5')-ppApApA and purified RNase F will be needed to resolve this question. Presence of naturally occurring (3'-5')-oligoadenylate molecules has not been reported and such compounds may never exist in the cell. If such compounds are present in vivo, high concentrations will be required for RNase F activation.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Stiftung Volkswagenwerk to A.P. We thank

Dr D. Wreschner for the stimulating discussions. G.A. was supported by the Foundation for Absorption in Science.

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