2′-Adenylated derivatives of Ap_3A activate RNase L

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Abstract The exact physiological function of Ap_3A (A5′ppp5″A, 5′5″ diadenosine triphosphate) remains unclear. Previously we have demonstrated that the human p46 2-5A synthetase (OAS1) efficiently utilises Ap_3A as an acceptor substrate for oligoadenylate synthesis. Here we show that $Ap_3A(2'p5'A)_n$ oligonucleotides can activate the 2-5A-dependent RNase (RNase L), when the number of 2′,5′-linked adenyl residues is two or more. Under the experimental conditions applied the half-maximal activation (AC_{50}) of RNase L for 2′-adenylated Ap_3A derivatives was determined to be in nanomolar range while the AC_{50} for 2-5A_3 was 0.4 nM. The $Ap_3A(2'p5'A)_n$ oligonucleotides are thus less effective in activating RNase L than 2-5A. We also investigated the occurrence of 2′-adenylated Ap_3A in interferon and poly(I)·poly(C)-treated HeLa cells. In purified trichloroacetic acid-soluble extracts about 40% of RNase L-activating material is resistant to phosphatase treatment, whereas the removal of 5′-terminal phosphates greatly reduces the activating properties of 2-5A. We assume that this activity at least partly may be associated with the presence of 2′-adenylated Ap_nA derivatives with blocked 5′-terminal phosphates.

Key words: RNase L; 2-5A synthetase; Ap_3A; (2′-5′)oligoadenylate; Interferon; Oligoadenylate synthesis; Poly(I)·poly(C)

1. Introduction

Activation of 2-5A system is one of the principal cellular effects of interferons (IFN) (reviewed in [1]). The IFN-inducible 2-5A synthetase is converted to catalytically active state by double-stranded RNA and polymerises ATP into 2′,5′-linked oligoadenylates (2-5A). The 2-5A oligomers bind to and activate RNase L that cleaves RNA at the 3′-side of UpNp sequences [2–4]. Activation of RNase L was shown to be important for cellular antiviral defence, in particular against picornaviruses [5]. Together with antiviral action the RNase L and the 2-5A systems in general were demonstrated to be involved in the regulation of cell growth and apoptosis [6].

It remains unclear whether 2-5A oligomers are the only physiological activators of RNase L and whether ATP polymerisation is the only function of 2-5A synthetases [7]. The level of 2-5A in cells of different types significantly increases after virus infection or treatment with cytokines and double-stranded RNA (poly(I)·poly(C)) [8–11]. Besides 2-5A, the occurrence of 2-5A-related compounds of unknown structure that differ from authentic oligoadenylates was observed in a number of IFN-treated and virus-infected cell lines [12–14]. These compounds were suggested to be alternative products of 2-5A synthetase that has relatively broad substrate specificity, being able to utilise as a primer any adenosine moiety with available 2′OH ribose group [14].

Previously we have shown that human 2-5A synthetase (p46 OAS) efficiently utilises Ap_3A, a dinucleotide with two symmetrical adenylate moieties, as an acceptor substrate for (2′-5′)oligoadenylate synthesis [15]. Ap_3A is a product of a reaction catalysed by aminoacyl-tRNA synthetases, in particular tryptophanyl-tRNA synthetase (WRS) [16]. A peculiar feature of the human WRS is its inducibility by IFN [17,18]. In parallel with WRS induction, the increase of Ap_3A level was demonstrated in cell lines of lymphocytic and monocytic origin after IFN treatment [19]. We suggested that IFN-dependent accumulation of Ap_3A, which is an effective primer for 2-5A synthetase, could promote cellular response towards viral invasion [15]. This study was aimed at finding experimental evidence for the ability of the $Ap_3A(2'p5'A)_n$ compounds to activate RNase L, which has not been investigated before, as well as the natural existence of 2′-adenylated Ap_3A derivatives.

2. Materials and methods

2.1. Baculovirus expression and purification of RNase L

Expression of RNase L in the baculovirus/insect system was done as described [3].

2.2. Sources of (2′-5′)oligonucleotides

2-5A and 2′-adenylated Ap_3A oligonucleotides were produced and purified as described [17].

2.3. Assay for RNase L

Oligoribonucleotide substrate $C_{11}UUC_7$ (synthesised by DNA technology, Aarhus, Denmark) was radiolabeled at 3′ position with 5′-[^{32}P]-3′,5′ cytidine bisphosphate (Amersham, 3000 Ci/mmol) according to supplier's protocol in the presence of 10% dimethyl sulfoxide, followed by ethanol precipitation. Incubation mixture contained 15 mM HEPES, pH 7.6; 90 mM KCl; 4 mM $MgCl_2$; 2 mM DTT; 1 mM ATP; 2 μM $C_{11}UUC_7$-[^{32}P]Cp and 100 ng of crude RNase L preparation. Reaction was performed in a total volume of 20 μl at 25°C in the presence or absence of various (2′-5′)oligonucleotides, or 2 μl of HeLa cell extracts for 10–30 min as indicated. Reactions were quenched by adding 20 μl of gel loading buffer containing formamide and 10 mM EDTA. To avoid the loss of material due to oligonucleotide absorption to the polypropylene surfaces, reaction tubes and pipette tips were siliconised in 2% dimethyldichlorosilane solution in trichloroethane. For product analysis, a 10-μl aliquot of

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Abbreviations: AC_{50}, half-maximal activating concentration; Ap_3A, diadenosine triphosphate; 2-5A, (2′-5′)oligoadenylate; BAP, bovine alkaline phosphatase; FPLC, fast protein liquid chromatography; IFN, interferon; TCA, trichloroacetic acid; WRS, tryptophanyl-tRNA synthetase

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the quenched solution was electrophoresed on 12% acrylamide, 7 M urea sequencing gel. The bands corresponding to substrate and product were quantified using a PhosphorImager (Molecular Dynamics).

2.4. Cell culture and extract

HeLa cells were grown in DMEM supplemented with 10% calf newborn serum (Life Technologies) to approximately 50% confluence before induction with 500 U of IFNα and with 50 U of IFNγ for 24 h. poly(I)·poly(C) (Pharmacia) after sonication was added to a final concentration of 0.2 mg/ml. Cells were incubated in the presence of poly(I)·poly(C) for 90 min and washed with phosphate buffered saline (PBS), then 2 ml of 7% TCA was added immediately after washing, and cells were harvested. Insoluble material was removed by centrifugation (6000×*g* for 10 min), and TCA was extracted by 1,1,2-trichlorofluoroethane/trioctylamine (3:1 v/v) (Merck). After extraction the solution was loaded on a 6-ml Resource Q column (Pharmacia), equilibrated with 0.1 M NH_4HCO_3. The column was washed with 20 ml of 0.22 M NH_4HCO_3, and oligonucleotide-containing fraction was eluted with 0.7 M NH_4HCO_3. Glacial acetic acid (0.1 volume) was added to eluate and CO_2 was completely evaporated, and then the solution was applied to a C18 Sep-Pak column (Water Associates) equilibrated with water. Oligonucleotide-containing fraction was eluted with 1 ml methanol/water (1:1). The eluate was lyophilised and dissolved in water in a volume equivalent to 1:5 of initial packed cell volume, and pH was adjusted to 7.6 by 0.5 M KOH. Solution was cleaned from possible RNase contaminations by filtration through Biomax-10K column (Millipore) at 3000×*g* for 40 min. In an aliquot, bovine alkaline phosphatase (BAP, Boehringer Mannheim) was added at 0.15 U/ml final concentration and incubated at 37°C for 1 h followed by heat denaturation at 85°C for 10 min. In parallel probe, the reaction was monitored by addition of [^{32}P]Cp-labeled 2-5A_3 up to 0.1 μM and followed by PEI thin-layer chromatography as described [15].

3. Results

To determine if 2′-adenylated Ap_3A derivatives could activate RNase L, the crude extract of recombinant human RNase L from baculovirus-infected insect cells was used. The amount of RNase L in cell extract was 5% of the total soluble protein as estimated from polyacrylamide gel electrophoresis (not shown). RNase L activity was monitored by measuring the cleavage rate of [^{32}P]Cp-labeled synthetic oligoribonucleotide $C_{11}UUC_7$ after separation of the substrate and product on denaturing polyacrylamide gel. The RNase L-dependent cleavage of this substrate leads to the accumulation of $C_{11}UU(3'p)$ and C_7-[^{32}P]Cp oligonucleotides [20].

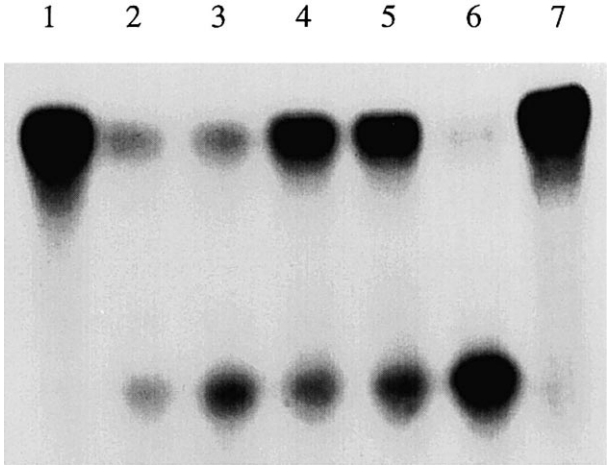

Fig. 1. Effects of 2′-adenylated Ap_3A derivatives on RNase L activity. Radiolabeled substrate ($C_{11}UUC_7$-[^{32}P]Cp) was incubated with RNase L at 25°C for 30 min, subjected to denaturing polyacrylamide gel electrophoresis and autoradiographed. Lane 1, 20 nM $Ap_3A(2'p5'A)$; lane 2, 20 nM $Ap_3A(2'p5'A)_2$; lane 3, 20 nM $Ap_3A(2'p5'A)_3$; lanes 4 and 5, 20 nM $Ap_3A(2'p5'A)_4$; lane 6, incubation in the presence of 5 nM 2-5A_3; lane 7, incubation without oligonucleotide activators.

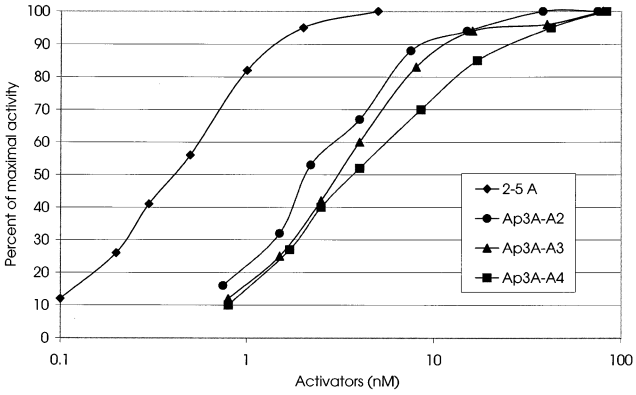

Fig. 2. Activation of RNase L as a function of oligonucleotide concentration. Assays were performed with 2 μM $C_{11}UUC_7$-[^{32}P]Cp as substrate at 25°C for 10 min. The rate of each reaction was determined relative to the maximal rate, which was obtained with the reactions incubated with 20 nM 2-5A_3 or 0.1 μM of each of the $Ap_3A(2'p5'A)_n$ respectively. The maximal activity obtained with $Ap_3A(2'p5'A)_4$ was 60% of that obtained with 2-5A.

Under the applied experimental conditions no basal RNase L activity was observed in the absence of specific oligonucleotide activators (Fig. 1, lane 1). In the presence of 5 nM 2-5A trimer [pppA(2′p5′A)$_2$] RNase L completely cleaves the substrate and a single radiolabeled product that is apparently C_7[^{32}P]Cp is formed (Fig. 1, lane 2). Oligonucleotides of $Ap_3A(2'p5'A)_n$ family are also able to activate RNase L with the exception of once 2′-adenylated Ap_3A (Fig. 1, lanes 3–6).

To determine RNase L-activating potential of Ap_3A derivatives, the rate of $C_{11}UUC_7$ cleavage was measured as a function of oligonucleotide concentration (Fig. 2). The rate of RNase L activity reached a plateau with increasing concentrations of oligonucleotides. At saturating concentrations the maximal activation of RNase L for $Ap_3A(2'p5'A)_4$ was about 0.6 from equipotent maximal activation for other tested oligoadenylates: 2-5A_3, $Ap_3A(2'p5'A)_2$ and $Ap_3A(2'p5'A)_3$. The $Ap_3A(2'p5'A)_2$ concentration at which half-maximal activation (AC_{50}) was obtained under the given reaction conditions was 2.8 nM. For 3 and 4 times 2′-adenylated Ap_3A derivatives the AC_{50} were 3.5 and 3.7 nM respectively. For the well-known RNase L activator 2-5A_3, the AC_{50} is 0.4 nM in agree-

Table 1
Activation of RNase L by various concentrations of $Ap_3A(2'p5'A)_2$ and oligonucleotides extracted from IFNα, IFNγ and poly(I)·poly(C)-treated HeLa cells (the autoradiogram is shown in Fig. 3)

Lanes	Activator	% Conversion
1, 2 and 19	No addition	0 ± 1.5
3	50 nM Ap_3A-2-5A_2	46.3
4	20 nM Ap_3A-2-5A_2	44.7
5 and 6	10 nM Ap_3A-2-5A_2	38.8 ± 4.0
7 and 8	5 nM Ap_3A-2-5A_2	31.0 ± 0.3
9 and 10	3 nM Ap_3A-2-5A_2	21.0 ± 2.5
11 and 12	2 nM Ap_3A-2-5A_2	10.5 ± 2.4
13 and 14	1 nM Ap_3A-2-5A_2	2.1 ± 0.4
15 and 16	Cell extract	26.1 ± 1.8
17 and 18	BAP-treated extract	11.4 ± 0.3

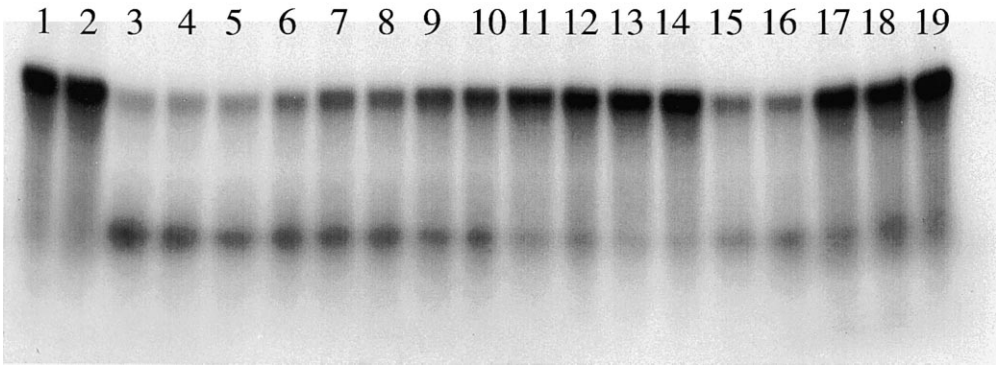

Fig. 3. Activation of RNase L by $Ap_3A(2'p5'A)_2$ and oligonucleotides extracted from IFN and poly(I)·poly(C)-treated HeLa cells. TCA-soluble extract was purified by FPLC, desalted by passing through Sep-Pak column, lyophilised and taken up in final dilution equivalent to 0.2 expected intracellular concentration. $C_{11}UUC_7$-[^{32}P]Cp as a substrate was incubated with RNase L at 25°C for 30 min and then electrophoresed and radioautographed. The RNase L activity in each lane was determined with a PhosphorImager and the calculated values after subtraction of background are shown in Table 1. This autoradiogram is representative of two–four independent experiments. Lanes 1, 2 and 19, incubation without activators; lane 3, 50 nM $Ap_3A(2'p5'A)_2$; lane 4, 20 nM $Ap_3A(2'p5'A)_2$; lanes 5 and 6, 10 nM $Ap_3A(2'p5'A)_2$; lanes 7 and 8, 5 nM $Ap_3A(2'p5'A)_2$; lanes 9 and 10, 3 nM $Ap_3A(2'p5'A)_2$; lanes 12 and 13, 2 nM $Ap_3A(2'p5'A)_2$; lanes 13 and 14, 1 nM $Ap_3A(2'p5'A)_2$; lanes 15 and 16, incubation in the presence of cell extract; lanes 17 and 18, incubation in the presence of BAP-treated cell extract.

ment with the published data [21,22]. Thus, $Ap_3A(2'p5'A)_2$, $Ap_3A(2'p5'A)_3$ and $Ap_3A(2'p5'A)_4$ were 8–10 times less efficient RNase L activators than (2′-5′)oligoadenylates.

Separately, we carried out experiments to detect possible occurrence of 2′-adenylated Ap_3A derivatives in IFN and poly(I)·poly(C)-treated HeLa cells. Purified extracts were diluted in water to a volume equivalent to 1:5 of the original packed cell volume and analysed for RNase L-activating capacity. Addition of this cell extract to the incubation mixture caused $C_{11}UUC_7$ cleavage at the same rate as cleavage induced by exogenous $Ap_3A(2'p5'A)_2$ at 4 nM concentration (Table 1, Fig. 3). Consequently, the average intracellular concentration of the RNase L-activating compounds could be roughly estimated as equivalent to 8 nM $Ap_3A(2'p5'A)_2$, or to 1 nM of 2-5A_3.

To ascertain the nature of these activators, we treated the extracts with BAP before RNase L assay. BAP treatment significantly reduced RNase L activation by TCA-soluble cell extracts (Fig. 3, lane 4). The BAP-resistant activating capacity was shown to be about 40% from the initial value and intracellular concentration could thus be estimated as equivalent to 3.2 nM of $Ap_3A(2'p5'A)_2$ or to 0.4 nM of authentic 2-5A_3. This remaining activity could be attributed to a cumulative action of unphosphorylated core 2-5A oligomers that retain residual activating capacity [2,3,22], and to 2′-oligoadenylated derivatives of Ap_3A (or Ap_4A) where 5′-terminal pyrophosphate group is protected from phosphatase action. The phosphatase-resistant activity cannot be attributed to endogenous cellular unphosphorylated 2-5A because these compounds were eliminated by an FPLC step.

4. Discussion

Diadenosine oligophosphates were suggested to be a peculiar class of both extracellular and intracellular effectors regulating a number of ion channels, membrane receptors, DNA replication and participating in various stress responses (reviewed in [23]). The IFN-dependent increase of the Ap_3A level in cultured cells correlates with the induction of WRS [19], which is known to synthesise Ap_3A from aminoacyl adenylate and ADP [24]. Besides, we have shown that Ap_3A serves as an acceptor substrate for human 2-5A synthetase (p46 OAS) which is capable of further elongating Ap_3A with up to six (2′-5′) adenylate residues [15]. This observation established a link between WRS and 2-5A synthetase, two of IFN-inducible enzymes.

There are two ways of 2-5A inactivation in vivo, namely dephosphorylation by cellular phosphatases or pyrophosphatases, and cleavage by specific 2′-phosphodiesterase [25]. The first way seems to be the major one. Analysis of oligonucleotide composition of TCA-soluble cell extracts from different tissues has shown that significant part of 2-5A oligomers are present at the unphosphorylated state [4]. Dephosphorylation greatly reduces the RNase L-activating capacity of 2-5A. Unphosphorylated 2-5A_3 and 2-5A_4 oligonucleotide cores are respectively 100 and 10 times less active than the 5′-triphosphorylated 2-5A_3 and 2-5A_4 [2,3,21]. In contrast to 2-5A, the pyrophosphate groups of 2′-adenylated Ap_3A derivatives are protected from phosphatase treatment by 5′-terminal adenosine moiety. It cannot be excluded that certain intracellular conditions, such as increase of Ap_3A concentration together with high level of phosphatase activity could lead to predominance of Ap_3A derivatives over authentic phosphorylated 2-5A. It is worth to note that general activity of 2-5A system in vivo depends from intracellular compartmentalisation of dsRNA-dependent 2-5A synthesis and RNase L targets, and from ratio between the levels of 2-5A synthesis and 2-5A dissipation that is a function of diffusion, hydrolysis and dephosphporylation [26]. One can suggest that accumulation of 2′-adenylated Ap_3A derivatives might direct RNase L towards other than standard 2-5A targets, which are more distant from dsRNA-containing intracellular compartments.

The following alternatives have already been discussed [15,23]: $Ap_3A(2'p5'A)_n$ compounds could activate RNase L directly, or this activation requires preliminary cleavage by specific Ap_3A hydrolase Fhit that presumably releases $ppA(2'p5'A)_n$ that activates RNase L when n is 3 or more. In this study we have demonstrated that 2′-adenylated Ap_3A derivatives themselves are able to activate RNase L in a nanomolar range of concentrations. We have determined that the

apparent AC_{50} for $Ap_3A(2'p5'A)_2$ is 2.8 nM whilst the apparent AC_{50} for trimer 2-5A is 0.4 nM. Before the apparent activation constant (K_a) for both 2-5A_3 and $Ap_4A(2'p5'A)_2$ was determined as 0.2 nM [21]. Hence, 2′-adenylated Ap_3A derivatives are less effective in RNase L activation in comparison with Ap_4A derivatives and authentic 2-5A oligomers, but at the same time Ap_3A is a more efficient substrate for 2-5A synthetase than Ap_4A [15].

The occurrence of 2′-adenylated Ap_3A derivatives in cells remains unclear. We demonstrated that oligonucleotide-containing fraction of TCA-soluble extract from IFN and poly(I)·poly(C)-treated HeLa cells retains partly the ability to activate RNase L after incubation with BAP. We suggested that this fraction is a complex mixture of 2′-adenylated Ap_3A (or Ap_4A) oligomers and unphosphorylated core 2-5A generated by BAP digestion. Our experiments demonstrate that the part of BAP-resistant component is about 40% from the total intracellular RNase L-activating material.

IFN-treated vaccinia virus-infected HeLa and several other cell cultures accumulate a complex mixture of authentic 2-5A, core 2-5A and numerous additional compounds [12–14]. In these studies HPLC of TCA extracts was combined with radiobinding assays. For a number of HPLC fractions the capacity to activate RNase L was resistant to phosphatase treatment. The chromatographic properties of these fractions were different from the dephosphorylated 2-5A [12–14]. Given that the chemical analysis of the HPLC fractions was not provided and the structure of other than 2-5A, the classical RNase L activators, remained unknown, it cannot be excluded that these compounds were 2′-adenylated Ap_3A (or Ap_4A) derivatives. In this case it is virus infection that stimulates significant accumulation of $Ap_3A(2'p5'A)_n$ oligomers in host cells in contrast to feeble accumulation after poly(I)·poly(C) treatment used in this study. It is worth to note that vaccinia virus is known to impair the action of 2-5A via inactivation of RNase L by a specific inhibitor and consequently with extremely high concentrations of intercellular 2-5A [27]. Further investigations are necessary to measure the exact cellular concentrations of 2′-adenylated Ap_3A derivatives and to unravel the physiological functions of these compounds.

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