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High yield synthesis, purification and characterisation of the RNase L activators 5′-triphosphate 2′–5′-oligoadenylates

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ABSTRACT

Upon viral infection, double-stranded viral RNA is detected very early in the host cell by several cellular 2′–5′ oligoadenylate synthetases, which synthesize 2′–5′ adenylate oligonucleotides that activate the cellular RNase L, firing an early primary antiviral response through self and non-self RNA cleavage. Transfecting cells with synthetic 2′–5′ adenylate oligonucleotides activate RNase L, and thus provide a useful shortcut to study the early steps of cellular and viral commitments into this pathway. Defined 2′–5′ adenylate oligonucleotides can be produced *in vitro*, but their controlled synthesis, purification, and characterisation have not been reported in detail. Here, we report a method suitable to produce large amounts of 2–5As of defined lengths *in vitro* using porcine OAS1 (pOAS) and human OAS2 (hOAS). We have synthesized a broad spectrum of 2–5As at the milligram scale and report an HPLC-purification and characterisation protocol with quantified yield for 2–5A of various lengths.

1. Introduction

The mammalian cell response to a viral infection is mainly mediated by type I interferon-regulated pathways (Sen, 2001; Servant et al., 2002). One of the main and early effects is to activate the 2′–5′ oligoadenylate synthetase (OAS)/ribonuclease L (RNase L) pathway (Roberts et al., 1976), where double-stranded non-self RNA produced during infection by the viral invader is recognized by one or several OAS isoforms. This enzyme is allosterically activated by dsRNA to synthesize small specific RNA molecules which are oligomers of adenosine with the particularity to be linked by 2′–5′ phosphodiester bonds instead of 3′–5′ as in "classic" RNAs (Hovanessian and Justesen, 2007). These oligomers (2–5As) are known to bind RNase L (Dong and Silverman, 1995), and the 2′–5′ phosphodiester bonds have been shown to be essential for the specific binding. The structural basis of this activation has been reported in the crystallographic structure of the 2–5A:RNase L complex (Tanaka et al., 2004), currently the only example of protein crystallized in complex with 2–5As. Once activated, RNase L cleaves both cellular and viral single-stranded RNA, thus playing an important role in the endogenous antiviral pathway (Silverman, 2007b). In recent years, several correlations between viral infections and the OAS/RNase L pathway have been reported (Behera et al., 2002; Kajaste-Rudnitski et al., 2006; Sawicki et al., 2003; Smith et al., 2005). Conversely, the ability of some viruses to counteract the antiviral action of RNase L has also been demonstrated (Han et al., 2007; Min and Krug, 2006; Xiang et al., 2002). Precise mechanisms remain ill-defined, though, and mechanisms of indirect or direct interaction between viral proteins and the OAS/RNase L pathway remain to be discovered.

No direct interaction of a viral protein with 2–5As has been reported yet. Conceptually, however, these 2–5As represent early triggers of the non-specific innate immune response, and thus, early viral interference with this pathway would be expected to efficiently dampen the host response to viral invasion. Indeed, direct induction of the RNase L response with these purified oligomers has been achieved successfully through transfection of 2–5A (Malathi et al., 2007; Silverman, 2007a). Likewise, activation of RNase L by small organic molecules has been shown to efficiently launch an antiviral state in the host cell (Thakur et al., 2007). Thus, it would be logic that viruses had evolved a way to interfere early with the innate immune response which involves the induction of more than a thousand actors in a potent and complex antiviral response.

With this idea in mind, we set up to obtain high amounts of pure and structurally characterised 2–5A in order to study their interaction with potential viral targets before they reach RNase L in the cell cytoplasm.

Several 2–5A synthesis protocols have been described, using either chemical or enzymatic method (Imai and Torrence, 1981;

Abbreviations: DP, degree of polymerisation; OAS, oligoadenylate synthetase; pOAS1, porcine OAS 1; hOAS2, human OAS 2; 2–5A, 2′–5′ oligoadenylate; RNase L, ribonuclease L; CIP, calf alkaline phosphatase; VCE, vaccinia virus capping enzyme; RNase T2, ribonuclease T2.

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Justesen et al., 1980; Kitade et al., 1991; Rusch et al., 2001; Thakur et al., 2007). However, the chemical synthesis of a 5′-triphosphate, a *sine qua non* condition to retain RNase L activation, is not easy and has been reported in an eight-step synthesis. In addition, neither easy nor large-scale production nor detailed purification, reporting yield, size distribution, and individual fractionation of size-controlled 2–5A, has been documented.

The enzymatic synthesis of 2–5A has been reported using purified OASs. Human OASs belong to a family of enzymes encoded by 3 closely linked genes with the following order: small (OAS1), medium (OAS2) and large (OAS3) isoforms, corresponding to proteins of 40/46, 69/71 and 100 kDa respectively. They are composed of one, two or three repeats of the basic OAS module, corresponding to their size (Hovanessian and Justesen, 2007). At their optimum activity conditions, OAS1 and OAS2 have the capacity to synthesize oligomers of "high" molecular weight, whereas OAS3 has the tendency to synthesize preferentially dimeric oligonucleotides. Some of these enzymes have been used to synthesize 2–5As (Rebouillat and Hovanessian, 1999), but no detailed protocol, precise purification, nor characterisation of the synthesized products has been described.

Here we report a protocol to produce large amount of different 2–5As *in vitro*. Two OASs, the porcine OAS1 (pOAS1) and the human OAS2 (hOAS2), were used and compared to synthesize a broad spectrum of 2–5As at the milligram scale with quantified yield for 2–5A of various lengths. We show that the Vaccinia virus mRNA capping enzyme is active as an RNA 5′-triphosphatase on the 2–5As, providing a simple and robust method to prepare 2–5A with a 5′-diphosphate. The purified 2–5As are able to induce rRNA degradation in transfected cell, as expected for an RNase L-mediated antiviral response.

2. Materials and methods

2.1. Expression and purification of recombinant human OAS2 in insect cells

The human OAS2 cDNA cloned in pFastBac vector (Invitrogen Bac-to-Bac Baculovirus Expression System) as a N-terminal His-tag fusion protein was a kind gift of Saumendra N. Sarkar and Games C. Sen from The Lerner Research Institute, Cleveland, USA. This construction was transformed into DH10-Bac *Escherichia coli* to produce bacmid DNA. The presence of recombinant bacmids was verified by PCR, and bacmids were used for the generation of human OAS2 recombinant baculovirus using Invitrogen Bac-to-Bac Baculovirus Expression System. All experiments were carried out according to the manufacturer's protocol in insect Sf9 cells. Sf9 insect cells were maintained in a spinner or monolayer cultures at 27 °C in Insect-XPress medium (BioWhittaker, Lonza). For large-scale production of the hOAS2 isoenzyme, 3×10^8 Sf9 cells were infected. 72 h post-infection, cells were harvested and the hOAS2 protein was purified as described in Sarkar and Sen (1998).

2.2. Expression and purification of recombinant porcine OAS1 in E. coli

The optimized gene of porcine OAS1 cloned into pET-15b as an N-terminal His-tag fusion protein was purchased from Geneart (Germany). The protein was expressed in BL21 (DE3) p*LysS* (Stratagene, U.S.A.) at 25 °C overnight after induction with 0.5 mM IPTG in LB Broth. The protein was purified in two steps, first with cation exchange chromatography (Cellulose Phosphate P11, Whatman) in 50 mM $NaPO_4$ buffer pH 7.5, 10% glycerol, 1 mM DTT, 0.1% Igepal CA630 (Sigma–Aldrich), 1 mM EDTA, and 50 mM NaCl. A gradient from 50 mM to 0.5 M NaCl was applied, and the protein was eluted at 0.32 M NaCl. Fractions were collected and applied to an immobilized metal affinity chromatography column (Ni-NTA, GE Healthcare) in 50 mM $NaPO_4$ buffer pH 7.5, 45 mM imidazole, 10% glycerol, 0.1% Igepal CA630, 1 mM EDTA, and 0.32 M NaCl. After washing with the same buffer, the protein was eluted in the same buffer supplemented with 240 mM imidazole (final concentration). The collected protein was dialysed against the same buffer without imidazole. The last dialysis buffer change constituted the storage buffer, i.e., 50 mM $NaPO_4$ pH 7.5, 50% glycerol, 0.3 M NaCl, 0.5 mM EDTA and 5 mM β-mercaptoethanol.

2.3. HPLC set-up

A Waters model 600 gradient HPLC system equipped with two 600 pumps, a 717 plus Autosampler injector, and a 996 photodiode array detector. An in-line degasser AF was employed for reverse phase chromatography. The separation column (Reverse Phase C18) and a pre-column (Reverse Phase C18) were installed in parallel on a two 7000 Rheodyne valve system (Interchim) for the online-cleaning procedure. A filter insert protected the column assembly. As buffer stock solution a 1 M solution of triethylammonium bicarbonate (TEAB) was prepared by adding dry-ice to a 1 M triethylamine solution until the pH reached 7.4 and filtered through 0.22 μM GV-type membranes (Millipore). HPLC eluents were freshly prepared. Eluent A was a 0.05 M solution of TEAB (pH 7.4) and eluent B was a 1:1 mixture of acetonitrile (HPLC grade, SDS, Peypin, France) and TEAB (final concentration 0.05 M, pH 7.4). The applied gradients for analytical and preparative separation of 2–5As are described in the corresponding sections (see below).

For HPLC coupled mass spectrometry, a Waters model Alliance 2790 system equipped with a photodiode array detector was employed. Mass spectra were recorded on an analyzer Quadrupole time-of-flight "Qtof" mass spectrometer (Waters). Conditions were accelerating potential 20 V, capillary potential 3000 V, source temperature 150 °C and nebulization temperature 250 °C.

2.3.1. Analytical scale

20 μl of sample was mixed with 180 μl of TEAB (0.05 M) and analysed. The column assembly consists of a pre-column (Delta-pak C18 100 Å, 5 μm, 3.9 × 20 mm) and a separation column (Nova-pak C18, 4 μm, 3.9 × 150 mm). Separations were run at a flow rate of 1 ml/min and started with a 5 min elution (100% eluent A) on the pre-column to remove proteic material. The analytical gradient started after 5 min at 100% eluent A with an increase to 8% eluent B after 15 min, to 15% eluent B after 30 min, to 20% eluent B after 35 min and to 100% eluent B after 40 min.

2.3.2. Preparative scale

250 μl of sample was mixed with 400 μl of TEAB (0.05 M) and analysed. The column assembly consists of a pre-column (XTerra prep MSC18, 10 μm, 10 × 10 mm) and a separation column (XTerra prep MSC18, 10 μm, 10 × 250 mm). Separations were run at a flow rate of 5 ml/min and started with a 5 min elution (100% eluent A) on the pre-column to remove proteic material. The analytical gradient started after 5 min at 100% eluent A with an increase to 8% eluent B after 15 min, to 15% eluent B after 30 min, to 20% eluent B after 35 min and to 100% eluent B after 40 min.

2.4. Mass spectrometry analysis

MALDI-TOF mass spectra were recorded on a Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) equipped with an N_2 laser (337 nm). MALDI conditions were accelerating potential, 24,000 V; guide wire, 0.05% of accelerating voltage; grid voltage, 94% of accelerating voltage; delay extraction time, 550 ns. Spectra were obtained in negative mode and were