Ap_3A and Ap_4A are primers for oligoadenylate synthesis catalyzed by interferon-inducible 2-5A synthetase

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Abstract The biological role of Ap_3A synthesized in cells by tryptophanyl-tRNA synthetase (WRS) is unknown. Previously we have demonstrated that the cellular level of Ap_3A significantly increases after interferon treatment. Here we show that the human 46 kDa 2-5A synthetase efficiently utilizes Ap_3A as a primer for oligoadenylate synthesis. The K_m for Ap_3A is several-fold lower than for Ap_4A and 100-fold lower than for ATP. This implies that Ap_3A might be a natural primer for the 2′-adenylation reaction catalysed by 2-5A synthetase. Since WRS and 2-5A synthetase are both interferon-inducible proteins, a new link between two interferon-dependent enzymes is established.

Key words: Tryptophanyl-tRNA synthetase; 2-5A synthetase; Ap_3A; Ap_4A

1. Introduction

It is well known that aminoacyl-tRNA synthetases (aaRS) catalyze the formation of aminoacyladenylates (for review see [1]). In the presence of cognate tRNA the activated amino acyl residue is transferred to the 3′ terminal ribose of tRNA or in excess of ATP the enzyme-bound aminoacyl adenylate is converted to Ap_4A (AppppA, diadenosine 5′,5‴-P^1,P^4-tetraphosphate). The Ap_4A synthesis is catalyzed by a series of bacterial and mammalian aaRS (for review see [2]). Contrary to the majority of aaRS, tryptophanyl-tRNA synthetase (WRS) fails to promote Ap_4A synthesis [3]. Instead, it catalyses the diadenosine 5′,5‴-P^1,P^3-triphosphate (Ap_3A, AppA) synthesis in the presence of ADP [4]. The cellular function of the Ap_3A remains obscure. Specific Ap_3A/Ap_4A asymmetrical hydrolase, called FHIT, was recently identified, sequenced and characterized in human cells [5,6].

Another peculiar property of the human WRS that clearly distinguishes this aaRS from all other aaRS is its interferon (IFN) inducibility [7–9] due to the presence of IFN-responsive regulatory elements GAS and ISRE in the promoter region of its gene [10–12]. The relation between the cell response towards IFN and the increased levels of WRS activity remains obscure. Several hypotheses have been proposed to accommodate this unusual behavior of WRS, a housekeeping enzyme, and the IFN action [7,8,13–16]. One of them suggests that as a consequence of the WRS induction, IFNs may promote accumulation of Ap_3A that may serve as an intermediate in the IFN signal transduction [17]. In fact, the Ap_3A accumulation was demonstrated experimentally in cell cultures of monocytic and lymphocytic origin [18]. In an attempt to find the potential target(s) of Ap_3A action we assumed that Ap_3A may serve as a substrate for another IFN-inducible enzyme, 2-5A synthetase [19–21]. This enzyme produces 2-5A from ATP by non-processive mechanism [21,22]. It has been proposed that 2-5A synthetase exhibits two binding sites, one for the primer (the acceptor) of the 2′-adenylation reaction, and one for ATP (donor) [22]. Besides 2-5A and ATP, 2-5A synthetase can adenylate several other nucleotides and oligonucleotides [23,24], and all ribonucleoside triphosphates can serve as donors. The only prerequisite for being an acceptor is the availability of the ribose 2′-OH group as in ATP, NADH, tRNA, ApA, CpA, etc. Since the symmetric diadenosineoligophosphates meet this requirement, we assume that Ap_3A and Ap_4A could serve as substrates for 2-5A synthetase. The aim of this work was to prove experimentally this assumption.

2. Materials and methods

2.1. *Purification of enzyme from Hi Five cells*

Hi Five cells infected for 72 h with a recombinant AcMNPV (Pia Møller Martensen, personal communication) containing a cDNA for 2-5A synthetase (46 kDa form) were lysed on ice for 10 min in 20 mM Tris-HCl, pH 7.5; 0.5 M $K(CH_3COO)$; 5 mM $Mg(CH_3COO)_2$; 1 mM EDTA; 1% Nonidet-40; 2% glycerol. The cell extract was cleared by centrifugation. Ammonium sulfate was added to the cleared cell extract to a final concentration of 1.1 M, incubated for 30 min on ice, and then centrifuged for 10 min at $20000 \times g$.

Ammonium sulfate was added to the supernatant to a final concentration of 1.7 M, loaded on a phenyl-Sepharose 6FF column (Pharmacia). The column was washed with a decreasing ammonium sulfate gradient (1.7–0 M). The proteins were eluted by ethylene glycol (gradient 0–50%). Peak fractions were identified by enzyme activity measurements using the spectrophotometric 2-5A synthetase assay as described [25]. The active fractions were subsequently loaded onto a MonoS column (Pharmacia, HR5/5) in 50 mM HEPES, pH 7.8; 50 mM $K(CH_3COO)$; 5 mM $Mg(KCH_3COO)_2$; 5% glycerol and eluted with increasing salt concentrations (linear gradient of $K(CH_3COO)$, 50 mM–1 M). Fractions with 2-5A synthetase activity were pooled and used for further analysis. Protein concentration was measured as described [26].

2.2. *Ap_3A adenylation by dATP*

Incubation mixture (final volume 50 μl) contained 20 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 0.2 mM DTT; 0.1 mM EDTA; 0.1 mg/ml BSA; 5% glycerol; 0.1 μCi of [α-^{32}P]dATP (Amersham); 0.5 mg/ml poly(I)·poly(C); 0.05 μg of 2-5A synthetase; dATP and Ap_3A (or Ap_4A) as indicated. The activity of 2-5A synthetase was determined by taking 10 μl aliquots after 10, 20, 30, 40 and 60 min of incubation at 25°C. After heat denaturation (90°C, 5 min) aliquots were treated with alkaline phosphatase (final concentration 0.3 U/ml) at 37°C for 3 h, and spotted onto PEI-cellulose plates. PEI chromatography was

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Abbreviations: aaRS, aminoacyl-tRNA synthetase; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; IFN, interferon; PEI, polyethylene imine; TLC, thin layer chromatography; WRS, tryptophanyl-tRNA synthetase

Enzymes: tryptophanyl-tRNA synthetase (EC 6.1.1.2), 2-5A synthetase (EC 2.7.7.-).

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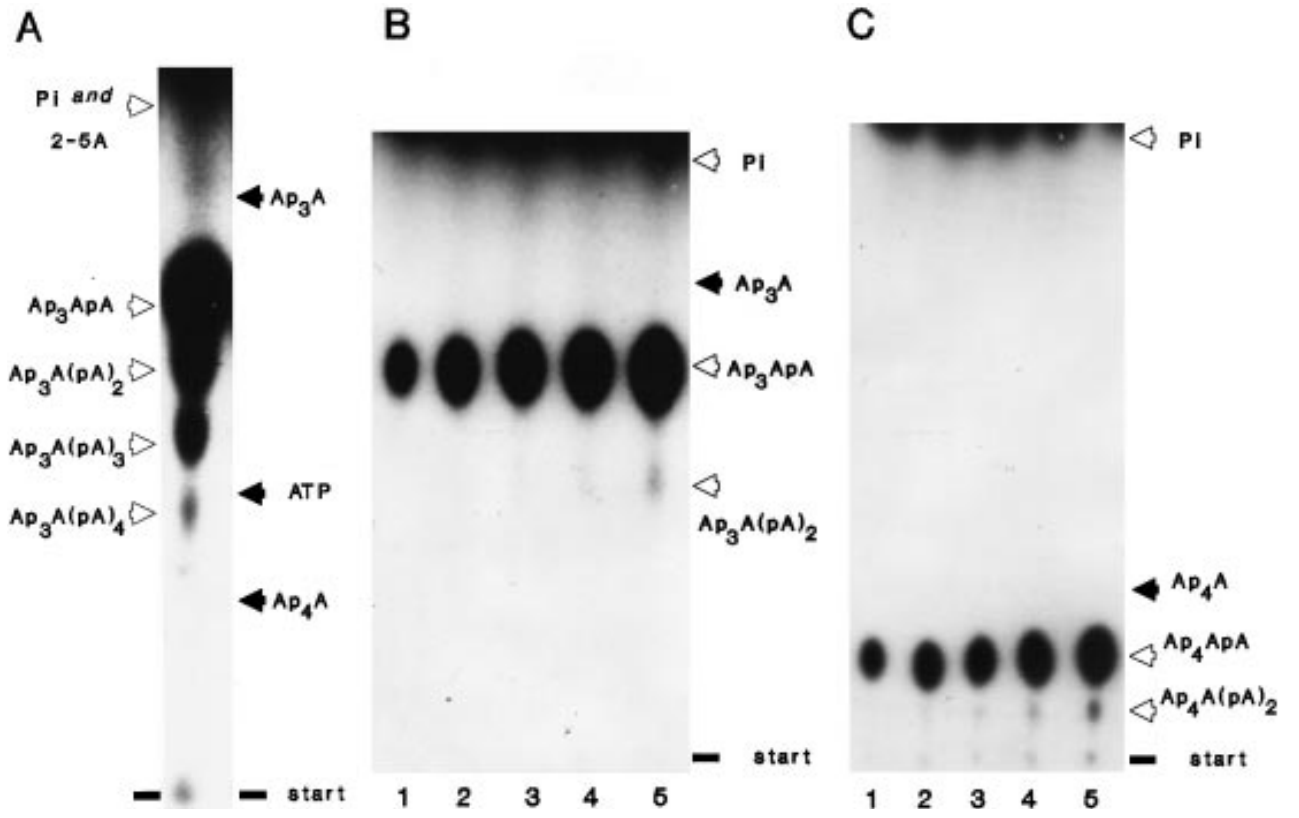

Fig. 1. Identification by PEI chromatography of Ap_3A and Ap_4A adenylation products. X-ray autoradiograms after exposition for 48 h. Filled arrows indicate mobility of the markers. A: Adenylation of Ap_3A by ATP. Incubation medium (50 μl) contained 20 μM Ap_3A, 0.2 μCi of [α-^{32}P]ATP, 400 μM ATP. Incubation for 1 h at 25°C. B: Time course for adenylation of Ap_3A by dATP. Incubation mixture contained 34 μM Ap_3A, 12 μM dATP. Incubation time: lane 1, 10 min; lane 2, 20 min; lane 3, 30 min; lane 4, 40 min; lane 5, 60 min. C: Time course for adenylation of Ap_4A by dATP. Incubation mix contained 60 μM Ap_4A, 12 μM dATP. Incubation time: lane 1, 10 min; lane 2, 20 min; lane 3, 30 min; lane 4, 40 min; lane 5, 60 min.

performed as described [20] using 2 M Tris-HCl, pH 8.83. ATP, ADP, Ap_3A, Ap_4A (Sigma) were used as reference standards. ^{32}P incorporation was measured by Phosphorimager (Molecular Dynamics) and respective bands quantitated by ImageQuant software. Autoradiograms were prepared by exposure of PEI-cellulose plates to X-ray films at −70°C with intensifying screen.

2.3. Ap_3A adenylation by ATP

Incubation mixture (final volume 100 μl) contained 20 mM Tris-HCl, pH 7.8; 10 mM $MgCl_2$; 0.2 mM DTT; 0.1 mM EDTA; 0.1 mg/ml BSA; 5% glycerol; 0.5 mg/ml poly(I)·poly(C); 6 mM creatine phosphate; 0.1 mg/ml creatine phosphokinase; 0.1 μg of 2-5A synthetase; Ap_3A [α-^{32}P]ATP and ATP as indicated. After incubation for 4 h at 37°C, reaction was stopped by heat denaturation and incubation mixture was treated with alkaline phosphatase before separation by TLC or FPLC.

2.4. FPLC analysis of oligonucleotides

The heat-inactivated reaction mixture (Section 2.3) was treated by alkaline phosphatase (Boehringer-Mannheim), diluted 10-fold in buffer A (20 mM Tris-HCl, pH 7.5) and applied to a MonoQ column (Pharmacia, HR5/5). The nucleotides were eluted by a linear salt gradient in buffer A (0–0.7 M NaCl) and detected at 254 nm. The flow rate was 0.5 ml/min, the volume of gradient was 25 ml. Analytical runs with and without alkaline phosphatase treatment and with a number of standard compounds (Ap_3A, Ap_4A, and ATP) were performed to establish the identity of the different compounds synthesized.

3. Results

That Ap_3A reacts with ATP in the presence of 2-5A synthetase and its activator poly(I)·poly(C) forming ApppA2′p5′A was first shown by TLC of radioactivity labelled nucleotides (Fig. 1A). The ApppA2′p5′A product remains a good substrate for 2-5A synthetase capable of reacting with another ATP molecule. The TLC shows that the initial product could be elongated with up to four additional AMP residues. However, with the resolution achieved by FPLC (MonoQ) of unlabelled molecules (Fig. 2A,B), up to 6 AMP residues per one Ap_3A primer could be visualized. The yield of the longer products is lower than for the shorter products. Since the Ap_3A molecule possesses two free 2′-OH groups, the di- and triadenylated Ap_3A are represented by two isomers, tetraadenylated Ap_3A is a mixture of 3 isomers, etc. The isomers can be identified by MonoQ column chromatography (Fig. 2B), where tri- and tetraadenylated Ap_3A each are visible as peaks with several summits.

To evaluate the substrate properties of diadenosineoligophosphates for 2-5A synthetase, kinetic analysis of the ApppA2′p5′dA synthesis was undertaken. In these experiments, dATP was used instead of ATP as donor in order to prevent 2-5A chain elongation. The adenylation yield depends linearly on dATP concentration in the range from 12 to 250 μM (data not shown). Reduction of the monoadenylated Ap_3A amount by its utilization for dA5′p2′ApppA2′p5′dA synthesis from ApppA2′p5′dA and dATP was only 2% (Fig. 1B) and this diminution was not taken into account in the calculations. Fig. 3A shows the results of kinetic measurements in dou-

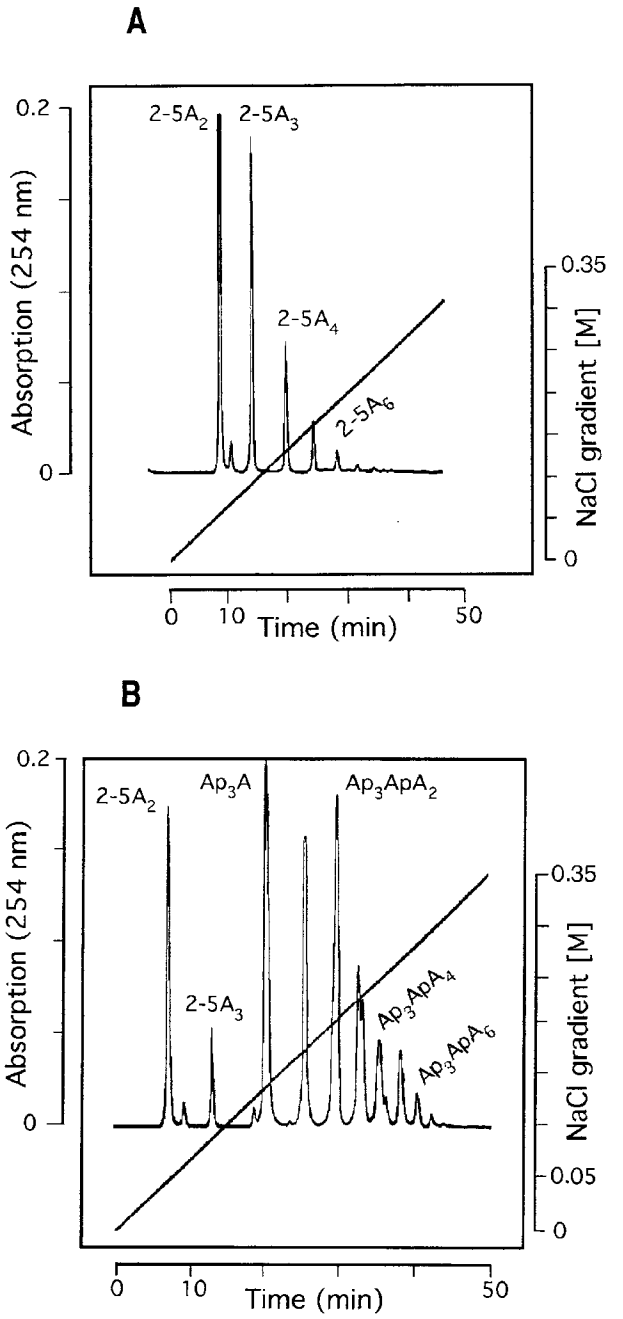

Fig. 2. Chromatography on MonoQ column of 2-5A synthetase products. Reaction conditions as described in Section 2.3. A: Separation of 2-5-oligoadenylates. Incubation mixture contained 1 mM ATP. B: Separation of Ap_3A adenylation products. Incubation mixture contained 1 mM ATP and 0.5 mM Ap_3A.

ble-reciprocal plots for the reaction rate and the Ap_3A concentration as the variable. Three fixed dATP concentrations were applied, 12, 60, and 200 μM. At these concentrations, K_m for Ap_3A was found to be 14.7, 18.0 and 71 μM, respec-

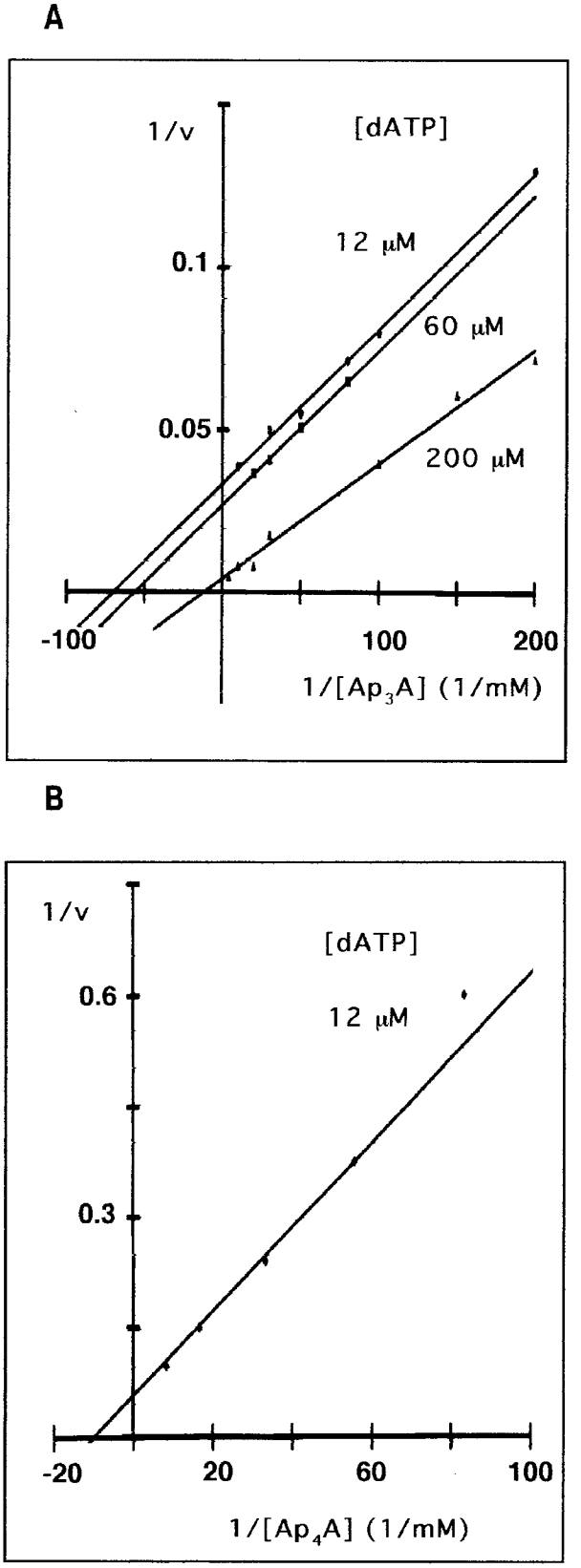

Fig. 3. The effect of Ap_3A/Ap_4A concentrations on 2-5A synthetase activity. Reactions were performed in the presence of dATP as described in Section 2.2. Double-reciprocal plots represent the variation in rate of Ap_3A/Ap_4A adenylation with initial concentrations of these substrates. Enzyme activity is expressed in nmol of adenylated Ap_3A/Ap_4A per min, per mg of protein. A: Inverse data plot for reaction of $Ap_3A2'p5'dA$ synthesis. The values of fixed dATP concentrations for each line are indicated on the figure. B: Inverse data plot for reaction of $Ap_4A2'p5'A$ synthesis. The fixed concentration of dATP was 12 μM.

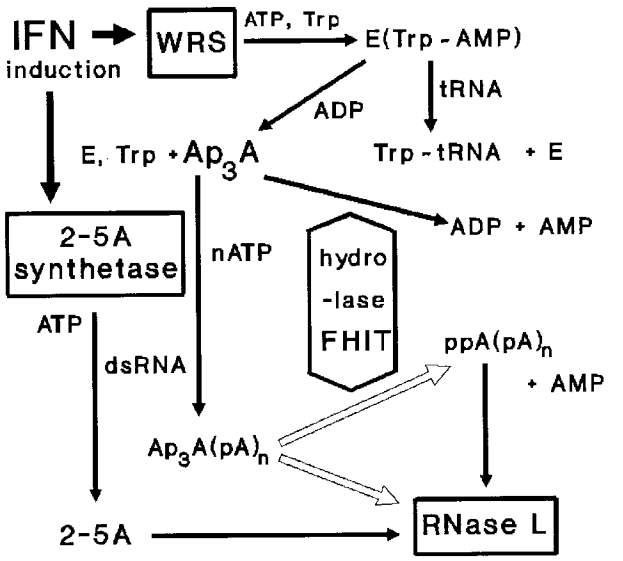

Fig. 4. Schematic representation of the Ap_3A metabolism and regulation pathways. Open arrows indicate proposed reactions. RNase L is activated by nanomolar levels of 2-5A resulting in breakdown of RNA in cells. We suggest that 2′-adenylated Ap_3A serves as modulator of RNase L. We also suggest the possibility that Ap_3A hydrolase FHIT cleaves $Ap_3A(2'p5'A)_n$ resulting in production of $ppA(2'p5'A)_n$ and following activation of RNase L.

tively. It is seen that two of these three curves are parallel to each other at low dATP concentrations. This implies that the 2′-adenylation reaction follows a ping-pong mechanism. Presumably, pyrophosphate split from dATP leaves the enzyme before the binding of Ap_3A to the 2-5A synthetase. We also performed the same measurements for Ap_4A as a substrate at 12 μM ATP (Fig. 1C). Under these conditions, Km for Ap_4A was found to be 99 μM (Fig. 3B). Therefore, Km for Ap_3A is 7 fold lower than for Ap_4A under the same reaction conditions.

4. Discussion

Ap_3A and Ap_4A are products of a side reaction catalyzed by several aaRS, and so far these are the only enzymes that produce these diadenosinepolyphosphates. Ap_3A and Ap_4A have been proposed to be intracellular signalling molecules which regulate DNA synthesis and stress responses [2]. Extracellular signalling function (binding to receptors of purines) of these nucleotides in blood and in neural secretory tissues has been suggested [27,28], and of intracellular functions there exist hypotheses for growth stimulatory effects due to affinity for DNA polymerases [2,29]. In this work we have demonstrated that Ap_3A and Ap_4A serve as very good substrates for pure human 2-5A synthetase (46 kDa form). Both diadenosineoligophosphates are much better substrates for 2-5A synthetase than, for example, ATP (K_m 2 mM) [30,31]. Moreover, Ap_3A has several-fold better affinity towards the enzyme than Ap_4A. Thus, the K_m for Ap_3A is comparable with the basal concentration of this dinucleotide in mammalian cells [32]. This may indicate that Ap_3A is a specific natural substrate for 2-5A synthetase. Indirectly, this conclusion is strengthened by the fact that Ap_3A efficiently competes with ATP for 2-5-oligoadenylate formation (Fig. 1A,B). Moreover, 2-5A synthetase apart from 2′-adenylation of Ap_3A is capable of further elongating the Ap_3A up to at least six adenylate residues. Therefore, Ap_3A serves as a specific primer (acceptor) for 2-5A synthesis. We have thus established a link between WRS and 2-5A synthetase, two enzymes of the IFN system.

What is the cellular role of $Ap_3A(pA)_n$ synthesized by 2-5A synthetase? This new compound may bind to IFN-inducible RNase L known to be activated by $2\text{-}5(A)_n$ (when $n > 2$) [33,34] and serve as either its competitive inhibitor or an alternative activator. We can suggest that $Ap_3A(pA)_n$ serves as a potential substrate to the Ap_3A hydrolase FHIT. This human enzyme cleaves Ap_3A to AMP and ADP [5]. If the same reaction also proceeds with 2′-adenylated Ap_3A, then the products would be AMP and a 2′,5′-oligoadenylate, bearing a 5′-pyrophosphate group, $ppA(pA)_n$. When n is greater than 1, this compound can even in nanomolar concentrations activate RNase L [33,35]. Thus, IFN treatment causes accumulation in cells of a primer for 2-5A synthetase activity that subsequently could accelerate the cellular response to dsRNA-containing virus invasion. If 2-5A products are secreted, it may be important that the $Ap_3A(pA)_n$ is resistant to phosphatase activity, whereas the classical 2-5-oligoadenylates are very sensitive. This could point to a function of $Ap_3A(pA)_n$ as a extracellular signalling molecule in blood and other tissues. All reactions revealed in this work and predicted from our observations are schematically depicted in Fig. 4.

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References

[1] L. Kisselev, A. Wolfson, Progr Nucleic Acid Res Mol Biol 48 (1994) 83–142.
[2] P. Zamecnik, Anal Biochem 134 (1983) 1–10.
[3] L. Kisselev, Biochimie 75 (1993) 1027–1039.
[4] T. Merkulova, G. Kovaleva, L. Kisselev, FEBS Lett 350 (1994) 287–290.
[5] L.D. Barnes, P.N. Garrison, Z. Siprashvili, A. Guranowski, A.K. Robinson, S.W. Ingram, C.M. Croce, M. Ohta, K. Huebner, Biochemistry 35 (1996) 11529–11535.
[6] M. Ohta, H. Inoue, M.G. Coticelli, K. Kastury, R. Baffa, J. Palazzo, Z. Siprashvili, M. Mori, P. McCue, T. Druck, C. Croce, K. Huebner, Cell 84 (1996) 587–597.
[7] J. Fleckner, H.H. Rasmussen, J. Justesen, Proc Natl Acad Sci USA 88 (1991) 11520–11524.
[8] B. Rubin, S.L. Anderson, L. Ling, W.P. Tate, J Biol Chem 266 (1991) 24245–24248.
[9] L.Yu. Frolova, M.A. Sudomoina, A.Yu. Grigorieva, O.L. Zinovieva, L.L. Kisselev, Gene 109 (1991) 291–296.
[10] L. Frolova, A. Grigorieva, M. Sudomoina, L. Kisselev, Gene 128 (1993) 237–245.
[11] I. Strehlow, D. Seegert, C. Frick, F.C. Bange, C. Schindler, E.C. Böttger, T. Decker, J Biol Chem 268 (1993) 16590–16595.
[12] D. Seegert, I. Strehlow, D.E. Levy, T.J. Decker, J Biol Chem 269 (1994) 8590–8595.
[13] A.B. Tolstrup, A. Bejder, J. Fleckner, J. Justesen, J Biol Chem 270 (1995) 397–403.
[14] J. Fleckner, P.M. Martensen, A.B. Tolstrup, N.O. Kjeldgaard, J. Justesen, Cytokine 7 (1995) 70–77.
[15] T. Flohr, F.C. Bange, A. von Euch, M. Kiekenbeck, E.C. Böttger, Infect Immunol 60 (1992) 4418–4421.
[16] K.T. Turpaev, V.M. Zakhariev, I.V. Sokolova, A.N. Narovlyansky, A.M. Amchenkova, J. Justesen, L.Y. Frolova, Eur J Biochem 240 (1996) 732–737.
[17] L. Kisselev, L. Frolova, A.-L. Haenni, Trends Biochem Sci 18 (1993) 263–267.

[18] A.A. Vartanian, A.N. Narovlyansky, A.M. Amchenkova, K.T. Turpaev, L.L. Kisselev, FEBS Lett 381 (1996) 32–34.
[19] I.M. Kerr, R.E. Brown, Proc Natl Acad Sci USA 75 (1978) 256–280.
[20] J. Justesen, D. Ferbus, M.N. Thang, Nucleic Acids Res 8 (1980) 3073–3085.
[21] M.J. Johnson, P.F. Torrence, In: R.M. Friedman, editor, Interferon 3. Elsevier, Amsterdam, 1984 pp. 189–298.
[22] J. Justesen, D. Ferbus, M.N. Thang, Proc Natl Acad Sci USA 77 (1980) 4618–4622.
[23] J. Justesen, H. Worm-Leonhard, D. Ferbus, H.U. Pedersen, Biochimie 67 (1985) 651–655.
[24] D. Ferbus, J. Justesen, F. Besancon, M.N. Thang, Biochem Biophys Res Commun 100 (1981) 847–856.
[25] J. Justesen, N.O. Kjeldgaard, Anal Biochem 207 (1992) 90–93.
[26] M. Bradford, Anal Biochem 72 (1976) 248–254.
[27] L. Gasmi, A.G. McLennan, S.W. Edwards, Blood 87 (1996) 3442–3449.
[28] H. Flodgaard, H. Klenow, Biochem J 208 (1982) 737–742.
[29] C. Weinmann-Dorsch, A. Hedl, I. Grummt, W. Albert, F.-J. Ferdinand, R.R. Friis, G. Pierron, W. Moll, F. Grummt, Eur J Biochem 138 (1984) 179–185.
[30] J. Justesen, D. Ferbus, M.N. Thang, Ann NY Acad Sci 350 (1980) 510–523.
[31] R.J. Suhadolnik, S.W. Li, R.W. Sobol, B. Haley, Biochemistry 27 (1988) 8846–8851.
[32] J. Luthje, A. Ogilvie, Biochem Biophys Res Commun 115 (1983) 253–260.
[33] D.H. Wreschner, T.C. James, R.H. Silverman, I.M. Kerr, Nucleic Acids Res 9 (1981) 1571–1581.
[34] R.H. Silverman, J Interferon Res 14 (1994) 101–104.
[35] K. Kariko, J. Ludwig, Biochem Biophys Res Commun 128 (1985) 695–698.