Activation of 2'-5' Oligoadenylate Synthetase by Single-stranded and Double-stranded RNA Aptamers*

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A number of small RNA molecules that are high affinity ligands for the 46-kDa form of human 2'-5' oligoadenylate synthetase have been identified by the SELEX method. Surface plasmon resonance analysis indicates that these RNAs bind to the enzyme with dissociation constants in the nanomolar range. Competition experiments indicate that the binding site for the small RNAs on the 2'-5' oligoadenylate synthetase molecule at least partially overlaps that for the synthetic double-stranded RNA, poly(I)poly(C). Several of the RNAs function as potent activators of 2'-5' oligoadenylate synthetase in vitro, although there is no correlation between binding affinity and ability to activate. The RNA aptamers having the strongest activation potential appear to have few base-paired regions. This suggests that 2'-5' oligoadenylate synthetase, which has previously been believed to be activated only by double-stranded RNA, can also be activated by RNA ligands with little secondary structure. Since 2'-5' oligoadenylate synthetase possesses no homology to other known RNA-binding proteins, the development of small specific ligands by SELEX should facilitate studies of RNA-protein interactions and may reveal novel features of the structure-function relationships involving this enzyme.

Double-stranded RNA (dsRNA) binds to and activates a number of interferon-induced proteins, namely the family of 2'-5' oligoadenylate synthetases (2-5A synthetase) and the protein kinase PKR (reviewed in Refs. 1–3). The synthetic dsRNA poly(I)poly(C) has been commonly used to stimulate these enzymes in vitro. In previous work, the interaction between 2-5A synthetase and the stimulatory dsRNA has been studied with a particular focus on poly(I)poly(C) (and modifications thereof), showing that a 10-base pair ideal helix in a 60-nucleotide-long dsRNA was necessary for the stimulation of 2-5A synthetase activity (4, 5). However, the RNA species used in those pioneering studies were all synthetic polymers not having a well defined length or structure.

Several small viral RNAs such as VAI encoded by adenovirus and the TAR RNA of HIV-1 have been found to activate 2–5A synthetase (6, 7), but inhibit PKR (8, 9), whereas the Rex response element from HTLV activates both 2–5A synthetase and PKR (10). It was shown recently that mutating the VAI RNA in a way that induced extra base pairing in the stem structure of this RNA increased the activation of 2–5A synthetase by VAI (6).

Many dsRNA-binding proteins, including PKR, possess dsRNA binding domains (dsRBMs) with related structures, and consensus sequences for these structures have been proposed (Refs. 11 and 12; reviewed in Ref. 13). However, despite the fact that the members of the 2–5A synthetase family are in general believed to be activated by similar types of RNA to those that activate PKR, the structural basis of the RNA-protein interactions in these enzymes might be rather different. Notably, the 2–5A synthetase family does not contain characteristic dsRBMs (14). For example, the binding of TAR to PKR involves interaction of the minor groove of the double-stranded helix with a dsRNA binding motif (dsRBM) (15). However, this motif is not present in any of the known 2–5A synthetase sequences, and no other structural similarity has been found between PKR and 2–5A synthetase. Several other RNA binding motifs have been identified over the last few years in both cellular and viral proteins (16). No obvious homology is found between these proteins and 2–5A synthetase, but deletion mapping has suggested that the RNA binding domain of 2–5A synthetase is situated in the N-terminal region of the protein (17).

To investigate the nature of the interaction between 2–5A synthetase and its activating RNAs, we have produced the 46-kDa form of human 2–5A synthetase in a baculovirus expression system and purified it from the infected insect cells. Subsequently, we tested the stimulation of enzymatic activity using a number of artificial aptamers selected through a SELEX procedure from a population of RNA molecules of 103 nucleotides in length containing internal sequences of 34 random nucleotides. We report the identification of small well characterized RNA molecules, which activate 2–5A synthetase at lower concentrations than any previously reported RNAs. Despite the absence of extensive secondary structure in these RNA aptamers, they function as potent activators of 2–5A synthetase in vitro. Annealing of complementary RNAs to the single-stranded aptamers increased the ability of these molecules to activate the enzyme in a sequence-specific manner.
Materials and Methods

Baculovirus Expression of Human 2′-5′ Oligoadenylate Synthetase

Complementary DNAs for the 1.6- and 1.8-kilobase forms of 2–5A synthetase (18, 19) were cloned into the EcoRI site of the baculovirus transfer vector pVL1393. Co-transfection with CsCl-purified recombinant transfer vector and linearized baculovirus (Autographa californica multiple nuclear polyhedrosis virus) DNA was performed as described by the suppliers (BaculoGold, Pharmingen). Spodoptera frugiperda (Sf9) cells (from ATCC CRL1711) were grown in Ex-Cell 401 medium (Sera Lab), supplemented with 5% fetal calf serum and antibiotics (100 international units/ml penicillin and 50 μg/ml streptomycin) (Life Technologies, Inc.), and adapted to growth in suspension in roller flasks.

Pure recombinant viruses were isolated using either plaque purification or a virus dilution method. Insect cells in 96-well microtiter plates were infected with serial dilutions of transfection solution, and cellular DNA was then examined for the presence of the 2–5A synthetase gene by dot blot analysis (20). Recombinant baculoviruses were tested for their ability to produce 2–5A synthetase by an enzymatic activity test (21).

Purification of Enzyme from High Five™ Cells

High Five™ (Trichoplusia ni from InVitrogen) cells were grown to 1.5 × 10^7 cells/ml and infected with recombinant virus in multiplicity of infection of 10. The infected cultures were harvested after 72 h by centrifugation at 13,000 rpm for 20 min. Large scale protein production was optimized in 400-ml roller flasks (Nunc, Roskilde, Denmark).

Cells were lysed on ice for 10 min in 1% Nonidet P-40 in buffer E (20 mM Tris-HCl, pH 7.5, 10 mM NaOAc, 10 mM Mg(OAc)2, and 1 mM EDTA). Cell extracts were cleared by centrifugation, and ammonium sulfate was added to a final concentration of 1.7 M. After incubation on ice and centrifugation for 10 min at 20,000 × g, ammonium sulfate was added to the supernatant to a final concentration of 1.1 M and the mixture was loaded on a Phenyl-Sepharose 6F column (Pharmacia Biotech Inc.). The column was washed with a decreasing ammonium sulfate gradient (1.7 M–0 M). The proteins were eluted with a gradient of 150 mM KCl, 10 mM MgCl2, 10 mM Hepes, pH 7.5, 200 mM potassium acetate, 2 mM MgCl2, 10 mM DTT).

Recombinant protein was from Dr. J. Kjemsa (IMS, University of Aarhus) and was prepared as described in Ref. 25.

Cloning and Sequencing—After either 8 or 11 rounds of selection, a portion of the RT-PCR product was cut with BamHI and HindIII and cloned into Bluescript-KS+ cloning vector (Stratagene). DNA was prepared from a number of colonies and sequenced using standard primers (M13–21 and M13 reverse). Sequencing was done automatically on an Applied Biosystems 373A sequencer, using the ABI Prism dye terminator cycle sequencing kit (Perkin-Elmer).

Preparation of Template for Antisense RNA Synthesis

The template was prepared by PCR, using a primer containing a T7 promoter at its 5′ end and 21 bases complementary to the 3′ end of the aptamer, and a primer identical to the 5′ end of the aptamer (antisense 5′ primer). The antisense RNA product was extended with an additional three G residues since the T7 RNA polymerase initiates most efficiently if the first three bases are GGG. The primer sequences were as follows: antisense T7 primer, 5′-TAATAC-GACCTCACTATAGGGGCGGGCGATCCGTTCATAATAGG-3′; antisense 5′ primer, 5′-GGGACCCACACCCACAT-3′.

Preparation and Purification of RNA

RNA was transcribed using the T7-MEGashortscript™ kit (Ambion). Templates were prepared by PCR, using the same primer as in the SELEX experiment. The transcription product was phenol-extracted, precipitated with ammonium acetate and isopropanol, washed with 80% ethanol, and redissolved in formamide loading buffer. After electrophoresis on a 6% sequencing gel, the RNA was visualized by UV shadowing. The RNA was then purified with a silica membrane (Ozyme), dried, and resuspended in 10 mM Tris-HCl, pH 8, 1 mM EDTA, and 0.1% SDS.

RPA Assay for 2–5A Synthetase Activity

The rapid pyrophosphate (RPA) assay for 2–5A synthetase activity was performed essentially as described in Ref. 27, except that buffer EM containing the coupling enzymes was prepared as two separate buffers. Buffer EMa contained 1.5 ml of 100 mM NADP+, 1 ml of 100 mM UDP-Glc, 150 μl of 10 mM glucose 1,6-bisphosphate, 15 ml of TM (70 mM triethanolamine base, 300 mM Mg(OAc)2, 20 mM KOAc, 2 mM EDTA, 1 ml of buffer EMb, and 11 ml of buffer TM with 50% glycerol). This mixture was added to a final concentration of 1.1 M ammonium sulfate. After heating to 94 °C for 5 min, followed by slow cooling to 37 °C, 10 μl of the RT-PCR reaction (28) was added to 10 μ1 of pure 2–5A synthetase (46-kDa form) and 35 μl of buffer EMa, 4 ml of buffer EMb, and 1 μl of buffer EM. The RPA assay was performed at 37 °C for 5 min, followed by slow cooling to 27 °C.
sigmoidal reaction velocity ($V_{max}$) were estimated using the following relations:

$$V = \frac{V_{max}}{1 + K_{app}/[RNA]}$$

where [RNA] is the concentration of the RNA agonist. Calculations were performed by making a non-linear curve fit of $V$ to [RNA] using SigmaPlot® software.

Thin Layer Chromatography of 2–5A Synthetase Products

2–5A synthetases were synthesized according to Ref. 21 using [α-32P]ATP and separated on polyethyleneimine-cellulose thin layer plates, developed with 2 M Tris-HCl, pH 8.63. Only intact triphosphorylated oligo-adenylates were detected, as well as the produced PPi.NaO

Filter Binding Assays

The filter binding assay was performed using [α-32P]UTP-labeled RNA. Approximately 50,000 cpm was incubated with the indicated concentration of 2–5A synthetase in binding buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 8 mM MgCl2, and 0.2 mg/ml RNase-free BSA (Boehringer Mannheim)) in a total volume of 25 μl for 15 min at 30 °C. 20 μl of the binding reaction was passed through a nitrocellulose membrane under vacuum. The membranes were washed three times with 300 μl of wash buffer (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 8 mM MgCl2). The stability of the RNA-protein complex was measured by diluting the binding reaction into 2 ml of wash buffer and incubating for various times prior to loading onto the nitrocellulose membrane. Complexes were stable for at least 10 min under these conditions.

Biosensor Measurements

All measurements were performed on a BIAcore 2000 instrument (Biosensor, Uppsala, Sweden) equipped with SA sensor chip. The SA sensor chip has a streptavidin-coated surface in all four flow channels (Biosensor, Uppsala, Sweden) equipped with SA sensor chip. The SA sensor chip has a streptavidin-coated surface in all four flow channels. Typically, 500–1000 response units (RU) of each RNA species was bound to the immobilized streptavidin corresponding to 0.18–0.022 pmol/mm².

Analyses of the 2–5A synthetase protein were performed by injecting 40 μl of protein solution into the derivatized sensor chip flow channels using a blank flow channel as a control. The protein was dissolved in Mg²⁺-HBS buffer and injected in concentrations of 40–600 nM. Generation of the sensor chip after each analysis cycle was performed by injecting 2 × 5 μl of 0.05% SDS. Kinetic parameters were determined by using the BIAevaluation 2.1 software.

Protein Synthesis in Rabbit Reticulocyte Lysates

Assays were performed as described in Ref. 28. The reaction volume was 10 μl. At the end of the incubation, 7 μl was taken for precipitation of acid-insoluble radioactivity, which was counted in a liquid scintillation counter.

RESULTS

Selection of RNA Ligands for 2–5A Synthetase—The 46-kDa isoform of 2–5A synthetase was used as the protein target in a SELEX experiment, using an RNA population of 103-mers containing randomized sequences within a 34-nucleotide stretch near the center of the molecule (Fig. 1B). RNA ligands for 2–5A synthetase were selected by either 8 or 11 rounds of selection using binding of the RNA-protein complexes on nitrocellulose filters. After elution of the RNA and amplification by RT-PCR, the products were cloned and sequenced. Out of a total of 20 aptamers obtained, two were identical (sx112 and sx118). Two others (sx106 and sx108) differ only at one nucleotide position. The sequences of the aptamers are shown in Fig. 2.

As a positive control for the procedure, selection for ligands with high affinity for the HIV-1 protein Rev was done in parallel, starting with RNA from the same stock. The sequences selected in this procedure corresponded to the three consensus motifs reported in Ref. 29 in a similar experiment (data not shown). This shows that the SELEX protocol used was valid for the purposes required.

Activation of 2–5A Synthetase by ssRNA or dsRNA Aptamers—Following the selection of aptamers by binding to 2–5A synthetase, RNA was prepared from the series of 20 aptamer clones and the ability of each RNA aptamer to activate 2–5A synthetase (46-kDa isoform) was tested in an in vitro assay.

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FIG. 1. Construction of the oligonucleotide library. A. Template construction scheme for the SELEX selection procedure. Oligonucleotides 1 and 2 were used to bridge the ligation of oligonucleotides 3 and 5 to oligonucleotide 4. Annealing oligonucleotide 1 to the ligation product creates a template for in vitro transcription using T7 RNA polymerase. Oligonucleotides 1 and 6 (amplification primers) contain HindIII and BamHI sites for cloning into the pBluescript-KS⁺ cloning vector after selection. B. Complete sequence of the transcripts containing the 34-mer random RNA inserts.
The activity observed in the presence of the different aptamers varied between 0.5- and 3.8-fold of the activity obtained with the random (preselection) RNA mixture. It should be noted that the RNA used in this initial screen was not gel-purified. Subsequently, we found that the activity stimulated by the random RNA decreased substantially if the RNA was gel-purified, as in the case of all subsequent experiments (see Table I). The activity stimulated by aptamers was not influenced significantly by the degree of purification.

A negative control using actin mRNA gave 12% of this activity. A positive control using poly(I)poly(C) gave a level of activation that was 2–3-fold of that of the random RNA (data not shown). The data presented in Fig. 3 is the result of a total of four experiments, using two independent RNA preparations.

**Alignment of Good agonists**

| Sequence | Description |
|----------|-------------|
| SX 137   | UCACUCUAAG---CCUACAAAG---UUGCACAAAG |
| SX 112/SX118 | ACACU---CCA---AAG---AAC---AUCAGAAAACUAGCUC |
| SX 131   | UCUGG-AUGACU-AG-3' |
| SX 96    | GCGACACAUACUACGA-ACGA-CCUGAU-3' |
| SX 101   | CTUAACUC-UCUACUAAGCUACUAG- ---UUAUC |

**Fig. 3. Abilities of selected aptamers to activate 2–5A synthetase.** Enzyme activity is expressed as reaction velocity, using a spectrophotometric assay, which allowed continuous measurement (see Fig. 4). Linear regression was applied within the linear range of the time courses generated. Assays contained 2 μg/ml RNA and 24 μg/ml 2–5A synthetase (46-kDa form) purified from baculovirus-infected insect cells. (Note that the RNA used in this experiment was not gel-purified, as was done in all subsequent experiments. This probably accounts for the relatively high activity of the control actin and random RNA transcripts; see “Results.”)
The aptamers were divided into groups with high, medium, and low activation potential. (The borders between these divisions were somewhat arbitrary, except that the random RNA was considered to define the low activity group). The results obtained in the initial screen were confirmed by making dilution series of selected aptamers, using a range of concentrations covering two orders of magnitude, as exemplified by the data for the sx112 aptamer shown in Fig. 4. Fig. 5 shows a comparison of three aptamers together with actin mRNA and random RNA, each assayed at three different RNA concentrations. 

The sx92, sx112, and sx137 aptamers showed a strong ability to activate the 2–5A synthetase, giving detectable activity at a concentration as low as 0.2 μg/ml in the case of sx112 (Fig. 4). At a concentration of 0.75 μg/ml, all three aptamers still exhibited a strong activity, whereas the control RNAs did not activate even at a concentration of 3 μg/ml. The results obtained in the RPA assay (Fig. 5) were confirmed in a direct assay using [α-32P]ATP with subsequent separation of the radioactive 2–5A products by thin layer chromatography on polyethyleneimine plates (21) (Fig. 6). No difference in the size distribution of the oligomers produced was observed whether poly(I)poly(C) or an aptamer was used as activator.

2–5A Synthetase Binding Assay—Aptamers from different groups (sx93, sx97, sx106, and sx112) were individually analyzed for binding to 2–5A synthetase by filter binding assays. All these RNAs bound to nitrocellulose filters in the presence, but not in the absence, of the enzyme. Fig. 7 shows the result obtained with the sx112 aptamer. This aptamer also bound to 2–5A synthetase in a concentration-dependent manner (data not shown), and binding was partially competed by poly(I)poly(C).

To further characterize the binding capability of the aptamer, we measured the dissociation constant for the RNA/2–5A synthetase complex using a Biacore machine. Four different aptamers and random RNA were biotinylated by incorporation of biotinylated UTP and subsequently bound to a streptavidin coated chips. The binding was analyzed by injecting 2–5A synthetase protein in five different concentrations ranging from 40 nM to 600 nM. The obtained kinetic parameters are presented in Table I. There is no apparent correlation between binding and activation. It is somewhat surprising that the random RNA binds 2–5A synthetase rather strongly; nevertheless, this might reflect a general RNA binding ability of 2–5A synthetase.

**Consensus Motifs in Activators of 2–5A Synthetase**—The three groups of aptamers (according to stimulatory activity) were compared at the sequence level (Fig. 2). In general, the selected aptamers have a high content of cytosine, and the presence of oligo(C) stretches is striking. This suggests an involvement of such sequences in the high activation of 2–5A synthetase by those RNAs. The strong agonists have a low content of G residues, with an average of only 3 G nucleotides in the 34 central randomized nucleotides. They are also characterized by two motifs, APyAPy(N)CC and UU(N)ACCC in different parts of the molecule. These motifs are also found in some of the medium and poor activators, but they are only partial (Fig. 2). We used the RNA DRAW computer program to examine possible secondary structures of the selected aptamers. A surprisingly low extent of base pairing was obtained for the aptamers, which were the best 2–5A synthetase agonists. The sx137 RNA had only a 3-base pair putative stem structure within the random region and the sx112 RNA had only a 6-base pair stem structure, which could be formed between the random region and part of the linker sequences (Fig. 8).

**Effects on Protein Synthesis**—In addition to the 2–5A synthetase another IFN-inducible enzyme, the protein kinase PKR, is also activated by binding structured RNA species. PKR inhibits protein synthesis by virtue of its ability to phosphorylate the α subunit of polypeptide chain initiation factor eIF2 (30). We have examined the ability of the most efficient 2–5A synthetase aptamer to activate PKR by monitoring protein synthesis in the rabbit reticulocyte lysate system, which contains endogenous latent PKR. Incubations were carried out in the presence and absence of 2-aminopurine. The latter inhibits PKR but has no effect on the activation of 2–5A synthetase by dsRNA. Thus, 2-aminopurine can be used to distinguish between inhibition of translation caused by PKR and that caused by the 2–5A/RNase L system (31, 32). The abilities of various concentrations of the sx112 aptamer to inhibit protein synthe-

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**Table I**

| Type of RNA | $K_{on}$ | $K_{off}$ | Activity |
|-----------------|--------|--------|--------|
| sx112 | $3.8 \times 10^4$ | $1.6 \times 10^{-4}$ | 0.3 |
| sx137 | $3.4 \times 10^4$ | $1.4 \times 10^{-4}$ | 0.19 |
| sx93 | $2.3 \times 10^4$ | $9.4 \times 10^{-5}$ | 0.05 |
| sx139 | $4.0 \times 10^4$ | $1.1 \times 10^{-4}$ | 0.06 |
| Random | $3.3 \times 10^4$ | $2.4 \times 10^{-4}$ | 0.02 |

**Fig. 4.** Time courses of 2–5A synthetase activity in the presence of different concentrations of the sx112 aptamer RNA. Enzyme activity was measured by absorbancy at 340 nm using the rapid pyrophosphate assay. Note that activity can be detected with a concentration as low as 0.2 μg/ml. The plateau of activity at high RNA concentrations is due to depletion of substrate for the coupling reactions.
sis are shown in Fig. 9. Poly(I)poly(C) was used as a positive control, and the unselected random RNA was used as a negative control. Whereas poly(I)poly(C) at 1 μg/ml was a potent inhibitor, and most of its effect was blocked in the presence of 2-aminopurine, much higher concentrations of the sx112 aptamer were required to inhibit translation. Moreover, this inhibition was not reversed by 2-aminopurine. This suggests that sx112 is not a good activator of PKR and that its main effect on protein synthesis (observed at 5 μg/ml and above) is mediated by the activation of 2–5A synthetase. Addition of the random RNA resulted in 2-aminopurine-sensitive inhibition of protein synthesis, even at concentrations as low as 1 μg/ml, suggesting that there was significant PKR activation (presumably caused by double-stranded regions present in the total RNA population).

Effects of Single-stranded and Double-stranded Forms of the Aptamers on 2–5A Synthetase—Since the sx112 aptamer was predicted by computer analysis to have a largely single-stranded structure, we went on to investigate the activation of 2–5A synthetase by the dsRNA formed by annealing sx112

**Fig. 5.** Comparison of 2–5A synthetase activity stimulated by various concentrations of different aptamers or control RNAs. Enzyme activity was measured as described in Fig. 4 in the presence of three different concentrations of the RNA species indicated. A, 12.5 μg/ml; B, 3 μg/ml; C, 0.75 μg/ml.
Activation of 2′–5A Synthetase by ssRNA and dsRNA Aptamers

Fig. 6. Direct assay of 2′–5A synthetase activity. Recombinant human 2′–5A synthetase (46-kDa form) (10 μg/ml) was incubated with the indicated RNAs at 3 μg/ml or 12 μg/ml for 1 h in the presence of [α-32P]ATP, as described under "Materials and Methods." The labeled 2′-5′ oligoadenylate products were separated from the ATP by thin layer chromatography and subjected to autoradiography. The relative activity was calculated as nanomoles of ATP polymerized after 1 h of incubation. Quantification of the 2′–5A products was achieved using a phosphorimager. The mobility of the ATP substrate and 2′-5′ oligoadenylate products is indicated. Lanes 8 and 9 show samples incubated in the absence of added RNA or of both RNA and enzyme, respectively. r.l.rC indicates poly(I)poly(C).

Fig. 7. Binding of sx112 RNA to 2′–5A synthetase. RNA-protein binding was assayed by the nitrocellulose filter method. In all experiments, [32P]-labeled sx112 RNA was present at a final concentration of 6 nm (0.2 μg/ml). All binding reactions contained 0.2 mg/ml BSA. Binding of sx112 RNA to 2′–5A synthetase alone or in the presence of various competitors. Column 1, no 2′–5A synthetase added; columns 2–5, 10 μg/ml total protein. The following RNA competitors were added: column 3, 10-fold molar excess of unlabeled sx112 RNA (2 μg/ml); column 4, 100-fold molar excess of unlabeled sx112 RNA (20 μg/ml); column 5, 10 μg/ml poly(I)poly(C).

sense RNA to sx112 antisense RNA. A construct was made from which RNA antisense to the aptamer could be transcribed using T7 RNA polymerase. Sense and antisense RNA were annealed by the same method as used for normal renaturation of RNA. Annealing of the complementary RNA led to a mobility shift in native polyacrylamide gels (data not shown). Similar hybrids were also made from three additional aptamers, sx137 representing another strong agonist and sx95 and sx139 representing the weak agonists. A concentration of 3 μg/ml total RNA was used in each assay of enzyme activity (corresponding to 82 nm for the ssRNA and 41 nm for the dsRNA hybrids) and the results are shown in Table II.

In each case, the dsRNA was a better activator of 2′–5A synthetase than the corresponding ssRNA, but the relative difference between the aptamers was approximately the same whether a single-stranded or double-stranded version was used. This confirms the sequence specificity observed when testing the single-stranded aptamers. The ssRNA version of sx112 stimulated activity, which was comparable to that produced by a 103-nucleotide-long, perfectly base-paired, double-stranded RNA prepared from sx95. The antisense RNAs alone always induced lower activity than the sense RNAs. This indicates that contaminating dsRNA is unlikely to be the cause of enzyme activation, since if that were the case, sense and antisense RNAs would be expected to give raise to similar activities. It is also evident from the effects of the hybrid dsRNAs that the constant arms, needed for amplification, can cause a low level of activation of 2′–5A synthetase (approximately 0.21–0.25 nmol of ATP/min) when present in double-stranded form.

To compare the kinetics of activation of 2′–5A synthetase by ssRNA and dsRNA, we estimated the apparent dissociation constant ($K_{app}$) and the maximal reaction velocity ($V_{max}$) for the enzyme in the presence of various RNA activators, with a 2′–5A synthetase concentration of approximately 0.75 μg/ml. The $K_{app}$ constant defines the concentration of RNA activator inducing half-maximal enzyme activity. Reaction velocity ($V$) was measured as the slope of the time course within the linear range, estimated by linear regression of at least four points. $K_{app}$ and $V_{max}$ were estimated using a non-linear curve fit of $V$ to the concentration of RNA. Dose-response curves are shown in Fig. 10, and the estimated parameters are summarized in Table III.

The ability of sx112 ssRNA to activate 2′–5A synthetase was apparently about 3.5-fold higher than that of poly(I)poly(C), based on the higher value for $K_{app}$ for the latter (measured in functional assays). However, we cannot exclude the possibility that the different $K_{app}$ values seen with poly(I)poly(C) and sx112 are a reflection of the different molecular weights of the two RNA species. In fact, poly(I)poly(C) is not very well characterized, but its average molecular weight is not thought to be larger than 200 kDa. Additionally, it is not known whether a poly(I)poly(C) molecule harbors one or multiple binding sites for 2′–5A synthetase.

Both poly(I)poly(C) and the dsRNA version of sx112 gave the same $V_{max}$, which was about 35% higher than the $V_{max}$ obtained with the ssRNA version of sx112. The fact that the ssRNA is incapable of reaching the same level of activation, even if added in saturating concentrations, as the dsRNA may suggest that the ssRNA aptamer traps a proportion of the enzyme molecules in an inactive conformation rather than an active one.

DISCUSSION

We have obtained a series of RNA aptamers using the SELEX method to select for binding to the 46-kDa form of human 2′–5A synthetase. We examined 20 of these aptamers for in vitro activation of 2′–5A synthetase using a simple assay previously developed in our laboratory (27) and have thereby identified a group of highly potent small RNA agonists of this enzyme. These agonists were also shown to be active in a direct assay of 2′–5A synthetase activity. The RNAs selected for their high affinity for 2′–5A synthetase are the strongest activators of this enzyme yet identified. Subsequently, we demonstrated that one of the most potent aptamers, sx112, is unlikely to be a strong activator of the dsRNA-dependent protein kinase PKR. This suggests a difference in the nature of the RNA structures necessary to activate 2′–5A synthetase and PKR.

For 2′–5A synthetase, no particular RNA species has been identified as a natural activator. However, certain small viral RNAs, such as VA RNA of adenovirus, Rex RNA of HTLV-1, and...
and TAR RNA of HIV-1, function as activators of the enzyme in vitro and have been suggested to act in this capacity upon viral infection (6, 7, 10). A similar effect has been observed in the case of the small RNA of Epstein-Barr Virus, EBER-1. The synthetic compound poly(I)\(\text{z}\)poly(C) is a widely used in vitro activator of 2–5A synthetase, but it is clearly not a physiological agonist for the enzyme in vivo. 2–5A synthetase is strongly stimulated by several other synthetic dsRNAs, including poly(I)\(\text{z}\)poly(C\(_{12}\)U\(_n\)) (Ampligen) and, to a lesser extent, poly(A)\(\text{z}\)poly(U) (5). In all cases, single-stranded homopolymers are inactive, and poly(G) is actually inhibitory. Double-stranded polymers containing a poly(C) stretch are generally the best activators, which is in agreement with our finding that all the selected aptamers were rich in C residues, whereas they

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**Fig. 8.** Computer-generated secondary structures of aptamers sx112 and sx137. The complete aptamer sequence was folded using the RNA Draw software; default parameters given by the program were used. The start and end of the randomized region are marked by squares. A, the sx112 aptamer; B, the sx137 aptamer.

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2 T. V. Sharp and M. J. Clemens, unpublished data.

3 R. Hartmann and J. Justesen, unpublished results.
contained few G nucleotides.

The first step in the evaluation of a SELEX experiment is normally to search for a consensus motif in the selected aptamers. The consensus found in the experiments reported here is rather weak. However, we confirmed the validity of the selection protocol by selecting for RNAs with high affinity for the HIV-1 protein Rev, from which we obtained consensus sequences similar to the one previously published (29) (data not shown). Moreover, if one takes into consideration the nature of the protein under investigation, the absence of a long linear consensus sequence necessary for binding to 2–5A synthetase is not entirely unexpected. This enzyme does not show any primary sequence specificity for its activation, but rather is activated by a variety of structured RNA molecules. In this way, the enzyme meets the same challenges as other parts of the immune system in that it must recognize a variety of foreign molecules (in this case double-stranded RNAs), but must not be stimulated by endogenous RNA structures. Most other protein-RNA interactions previously analyzed in SELEX experiments have been based on specific interactions in vivo between a protein and an already well defined RNA element, such as phenylalanyl-tRNA synthetase and its cognate tRNA (33) or the HIV-1 Rev protein and the Rev-responsive element (29, 34). These considerations notwithstanding, two small consensus motifs were identified among the strong 2–5A synthetase agonists. There is no doubt that sequences within the unique regions of the aptamers are responsible for the strong and specific activation seen with this group, since other RNAs with the same common 5′ and 3′ sequences were poor activators.

The computer program RNA DRAW was used to predict the secondary structures of the aptamers belonging to the group of strong agonists. The longest potential double helix was found in sx131; this was 9 base pairs long but contained several internal bulges (data not shown). Surprisingly, the most active aptamer, sx112, had only a 3-base pair putative dsRNA stem within the central region (on which the SELEX selection is based). The accuracy of computer programs for predicting RNA secondary structure is uncertain, especially when a number of possible helices exist. However, the RNA DRAW program uses the minimal energy algorithm devised by Zuker (35), which

**FIG. 9.** Inhibition of protein synthesis by various RNAs in the rabbit reticulocyte lysate system. Protein synthesis was measured by the incorporation of [14C]leucine into acid-insoluble material as described under “Materials and Methods,” in the presence of various concentrations of the indicated RNA species. Incorporation in the control incubation without added RNAs was 51,000 cpm. Data are expressed as the percent inhibition of incorporation relative to this value. Filled bars, without addition of 2-aminopurine; hatched bars, with addition of 2-aminopurine (15 μM). The addition of 2-aminopurine alone had no effect on protein synthesis (data not shown).

**TABLE II**

| Type of RNA | Activity Normalized to poly(I)poly(C) |
|-------------|-------------------------------------|
| sx112 sense | 0.31                                |
| sx112 antisense | 0.10                                |
| sx112 sense + antisense | 1.35                                |
| sx137 sense | 0.19                                |
| sx137 antisense | 0.13                                |
| sx137 sense + antisense | 1.67                                |
| sx95 sense | 0.05                                |
| sx95 antisense | 0.03                                |
| sx95 sense + antisense | 0.31                                |
| sx139 sense | 0.06                                |
| sx139 antisense | 0.04                                |
| sx139 sense + antisense | 0.82                                |
| sx112 sense + sx95 antisense | 0.21                                |
| sx112 sense + sx137 antisense | 0.25                                |
| sx95 sense + sx112 antisense | 0.24                                |
| sx139 sense + sx95 antisense | 0.21                                |
| Poly(I)poly(C) | 0.25                                |

**FIG. 10.** Analysis of activation of 2–5A synthetase by aptamer RNAs and poly(I)poly(C). 2–5A synthetase activity is shown as a function of the concentration of single-stranded sx112 RNA (open circles), double-stranded sx112 RNA (triangles), and poly(I)poly(C) (closed circles) on a log scale. The points represent the measured values, and the lines are the best fit curves.

**TABLE III**

| Type of RNA | Estimated kinetic parameters for various RNA activators |
|-------------|--------------------------------------------------------|
|             | K<sub>app</sub> (μg/ml) | V<sub>max</sub> (nmol ATP/min) |
| Poly(I)poly(C) | 42                  | 1.29                      |
| Single-stranded sx112 | 9.3                | 0.73                      |
| Double-stranded sx112 | 4.8               | 1.29                      |
searches for all possible base pairings and is more likely to overestimate than underestimate the actual number of base pairs within a given RNA molecule. We therefore conclude that the 2–5A synthetase activators described in this paper, despite being the strongest yet identified, have a predominantly single-stranded rather than the expected double-stranded structure. At first sight, this is a surprising conclusion, but it is supported by the fact that one of the strongest agonists, the ssx12 aptamer, was a poor activator of the dsRNA-dependent enzyme PKR. In contrast, the random RNA pool used in the SELEX procedure was apparently able to activate PKR, suggesting the presence of dsRNA molecules in the pre-selection RNA population.

PKR and 2–5A synthetase have no significant similarities in their amino acid sequences, and the consensus dsRBM motif that is present in many dsRNA-binding proteins (11, 12) is notably absent from the latter enzyme (14). Furthermore, 2–5A synthetase possesses no known homology to other RNA-binding proteins. It is therefore likely that the region of the 2–5A synthetase protein interacting with RNA is quite distinct from that of PKR and recognizes different RNA structural motifs. On the basis of the data obtained in this paper and the lack of sequence similarity, we therefore suggest that PKR and 2–5A synthetase interact with RNA molecules through different structural mechanisms.

Annealing complementary RNA to different aptamers led to an increase in the stimulation of 2–5A synthetase. The ratio of stimulation between the good and bad aptamers was roughly the same whether ssRNA or dsRNA versions were used. Since the ssx12 ssRNA had a stimulatory ability comparable with perfectly base-paired dsRNA forms of the otherwise weakly activating aptamers, the strong activation seen with ssx12 appears to arise from interactions between the enzyme and a motif within the ssx12 sense strand alone. Annealing complementary RNAs to aptamer RNAs, converting them into normal Watson-Crick base-paired helices, probably eliminates all previous secondary structures that were present. Nevertheless, it promotes activation of the synthetase in a sequence-specific manner, leading us to favor a model where the protein interacts directly with bases in the sense strand.

We estimated the $K_{\text{app}}$ and the $V_{\text{max}}$ for single- and double-stranded versions of ssx12 RNA, and for poly(I)-poly(C), as activators of 2–5A synthetase. The $K_{\text{app}}$ determined for ssx12 ssRNA was about 4 times lower (9.3 μg/ml) than for poly(I)-poly(C) (42 μg/ml), but the $V_{\text{max}}$ found with single-stranded ssx12 RNA was lower than that with poly(I)-poly(C) or double-stranded ssx12 RNA.

We propose an interpretation of the kinetic data according to the “two-state model” of Del Castillo and Katz, reviewed in Ref. 36. This model has widespread applicability in describing the activation of receptors by agonists, but can equally well be applied to allosteric activation of enzymes. In short the model characterizes an agonist by two dissociation constants rather than one, $K_a^*$ being the dissociation constant for the active state ($E^*$) of the enzyme and $K_i^*$ being the dissociation constant for the inactive state of the enzyme ($E$). The efficacy of an agonist then depends on the ratio of $K_a^*$ to $K_i^*$ (i.e. if $K_a^* / K_i^*$ is above 1, then the molecule behaves as an agonist, the higher the ratio the more effective the agonist is). In our case, the ssx12 ssRNA has an lower efficiency for activation of 2–5A synthetase than the corresponding dsRNA. However, it is reasonable that a ssRNA aptamer might bind well to both active and inactive forms of the enzyme (resulting in a relatively low efficiency of activation), since it is selected solely upon its ability to bind 2–5A synthetase.

In a given system, the maximal fraction of an enzyme trapped in the active state by an agonist saturating concentration is then determined solely upon the ratio of $K_a^* / K_i^*$. According to this model, an agonist can have a high affinity for the enzyme, inducing activity at low concentrations, but still leading to a lower $V_{\text{max}}$ as seen for the single-stranded ssx12. It is not surprising that an aptamer selected by its ability to bind to the protein under conditions of competition for a limited number of binding sites has a high affinity for the inactive state as well as the active state. The dsRNA agonists have a higher efficacy than single-stranded ssx12, as indicated by the higher $V_{\text{max}}$ achieved, implying that dsRNA structures preferentially interact with the active state of 2–5A synthetase.

We found a large difference between the $K_a^*$ measured on the Biacore machine and the RNA concentration necessary to give half-maximal activity. A similar difference in binding and activation was found in Ref. 10 using UV cross-linking as the method for measuring $K_a^*$. This implies that the mechanism of activation of 2–5A synthetase is more complex than the simple binding of a suitable RNA ligand.

Although dsRNA has been demonstrated to be the preferred activator of 2–5A synthetase, the ssRNA molecules described here activated the enzyme as strongly as any previously described dsRNA agonist. However, other dsRNA-dependent pathways, including the activation of PKR, may not be stimulated by these ssRNA molecules. Future experiments will be required to show whether the aptamers induce the biosynthesis of interferon as efficiently as does poly(I)-poly(C). Our well defined aptamers could be expressed in cells in a number of ways, thus giving valuable information on the in vivo activation and function of 2–5A synthetase in the absence of other dsRNA-sensitive events.

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