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Immunology

Monoclonal antibodies to 5′-triphospho-(2′-5′)adenyladenosine oligonucleotides

(radioimmunoassay/interferon/hybridoma)

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ABSTRACT **Thirteen monoclonal antibodies to ppp(A2′p5′)$_n$A oligonucleotides have been produced by fusing Y3 myeloma cells with spleen cells of rat hyperimmunized with A2′p5′A succinyl albumin as immunogen. ^{125}I-Labeled A2′p5′A succinyl tyrosine methyl ester was used as a labeled probe. Antibodies were detected by their ability to bind the labeled analog and were selected for their affinity for A2′p5′A. There were no significant differences between the properties of the monoclonal antibodies and of the antiserum. They discriminated between 2′-5′ and 3′-5′ phosphodiester bonds: they crossreacted poorly with (A3′p5′)$_2$A (crossreactivity ratio, $>10^4$) and even less with ATP and adenosine (crossreactivity ratio, $>10^6$). The affinity was high ($K_d = 6 \times 10^{-12}$ M) for succinyl A2′p5′A, which is the best ligand, and also high for A2′p5′A (crossreactivity ratio, 3) and (A2′p5′)$_2$A, (A2′p5′)$_3$A, and (A2′p5′)$_4$A (crossreactivity ratio, 7.5). The binding to triphosphorylated isomers, ppp(A2′p5′)$_n$A, was affected by the presence of the triphosphate groups, and the affinity increased as the length of the isomer increased (crossreactivity ratio, 10,000 for $n = 1$ to 200 for $n = 4$). Thermodynamic analysis of these data demonstrated that, in dephosphorylated isomers, the binding site of highest affinity was located at the 5′-OH end of the molecule whereas in phosphorylated isomers, the favorable binding sites were in the middle and at the 2′-OH end of the chain. Use of monoclonal antibodies of such a specificity together with the ^{125}I-labeled 2-5A analog allows quantification of (A2′p5′)$_n$A directly and of ppp(A2′p5′)$_n$A after removal of the terminal phosphates by alkaline phosphatase treatment.**

Triphospho(adenylyl 2′-5′)$_n$adenosine [ppp(A2′p5′)$_n$A, $1 < n < 5$; also referred to as 2-5 A] are oligonucleotides synthesized from ATP by a synthetase that is widely distributed in tissues and cells and whose activity increases in response to interferon and varies with growth and hormone treatment (1–5). ppp(A2′p5′)$_n$A inhibit protein synthesis at subnanomolar concentrations by activating a nuclease present in most mammalian cell types (6). They act in cell-free systems (7) and in permeable cells in which the nonphosphorylated cores (A2′p5′)$_n$A (also referred to as 2-5 A cores) are also biologically active (8–10).

Thus, the components of the ppp(A2′p5′)$_n$A system appear to be present in cells exposed not only to interferon but also to various physiological stimuli. Questions about its real importance and its exact role are still unresolved. Answers should come from the quantification of each oligomer of the family. Because of the low levels expected in cells, only competition binding assays are likely to fulfill the requirements of specificity and sensitivity. This raises three essential problems: (*i*) finding a binding protein with optimal affinity and specificity [nuclease and antiserum have already been proposed (11–12)]; (*ii*) providing a labeled probe that combines a high specific radioactivity with a sufficiently long half-life [this is not the case for ^{3}H or ^{32}P compounds (13)]; and (*iii*) resolving the specific problem due to the polymorphism of 2-5A (in other words, to make possible the quantification of all the oligomers at the same level of sensitivity).

Our solution was to develop monoclonal antibodies against A2′p5′A, the repetitive unit of the family, and to synthesize a ^{125}I-labeled analog. Here we describe the synthesis, purification, and characterization of the immunogen and the labeled antigen. We compare properties of the whole antiserum and 13 monoclonal antibodies. We demonstrate preferential antibody binding to the 5′-OH end of dephosphorylated isomers and to the 2′-OH end of the phosphorylated ones. We propose a radioimmunoassay which, combined with phosphatase treatment, allows determination of each oligomer at the same level of sensitivity.

MATERIALS AND METHODS

Materials. Adenylyl-2′,5′-adenosine, adenylyl-3′,5′-adenosine, AMP, ATP, and adenosine were purchased from Sigma. Adenylyl-2′,5′-adenylyl-2′,5′-adenosine and 5′-triphosphoadenylyl-2′,5′-adenylyl-2′,5′-adenosine were purchased from P-L Biochemicals. Phosphodiesterase (oligonucleate 5′-nucleotidohydrolase, EC 3.1.4.1) from *Crotalus durissus terrificus* was purchased from Boehringer-Mannheim (GFR) and alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], type III R from *Escherichia coli* was purchased from Sigma. Polyethylene glycol 6000 was obtained from Merck.

Enzymatic Synthesis of ppp(A2′p5′)$_n$A. HeLa cells treated with human fibroblast interferon were used as a source of ppp(A2′p5′)$_n$A synthetase as described (14).

Identity and purity of 2′,5′A oligomers were checked by using the following criteria: charge in Tris/urea chromatography on DEAE-Tris-Acryl; high-voltage paper electrophoresis at pH 1.85; HPLC retention time for dimer and trimer after extensive alkaline phosphatase digestion; differential sensitivity to T2 RNase; hyperchromicity at 260 nm after phosphodiesterase or alkaline hydrolysis. Molar extinction coefficients at 260 nm were taken as 25,800 for dimer, 36,000 for trimer, 41,600 for tetramer, and 50,000 for pentamer according to reported data (12, 15, 16) and our own measurements of hyperchromicity shifts during phosphodiesterase digestion.

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Abbreviations: (A2′p5′)$_n$A $1 < n < 4$, oligo (2′-5′ adenylyl)$_n$adenosine; ppp(A2′p5′)$_n$A $1 < n < 4$, 5′ triphospho-oligo (2′-5′ adenylyl)adenosine; Suc, succinyl.

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$(A2'p5')_nA$ with n = 1–4 were quantitatively derived from their phosphorylated analogs by alkaline phosphatase treatment.

Analytical Procedures. HPLC was performed on a weak anion-exchanger (μBondapak NH_2 column; 30 cm × 3.9 mm i.d.) from Waters Associates, equilibrated and eluted under isocratic conditions with 0.01 M potassium phosphate at pH 3. The flow rate was 1.4 ml/min; elution was monitored at 260 nm.

TLC was performed on fluorescent polyethyleneimine-cellulose with 1.25 M NaCl as solvent and on Polygram Cel 300 with ETOH/0.1 M CH_3COONH_4, 5:2 (vol/vol) as solvent. Tyrosine derivatives were detected by the nitrosonaphthol method (17) either in aqueous medium or on TLC.

Ester and phosphodiester bonds were cleaved with 1 M KOH at room temperature. Hydrolysis of ester bonds was instantaneous and complete; hydrolysis of phosphodiester bonds took several hours.

One volume of enzyme (phosphatase or phosphodiesterase) solution was mixed with 10 vol of sample in 1 M Tris·HCl, pH 9.3/10 mM $MgCl_2$ and incubated 30 min at 37°C.

Electrofocusing of antibodies was performed on PAG plates (pH 3–9). Antibodies were complexed with the ^{125}I-labeled probe and detected by autoradiography.

Derivation of Monoclonal Antibodies. Male rats (Lou/WSI) were immunized with 250 μg of 2′ and 3′ disuccinyl$(Suc)_2$A2′-p5′A complexed to human serum albumin (2 mol/mol). At 72 hr after the final injection, immune spleen cells were fused with Y3 myeloma cells and hybrid cells were selected as described (18, 19).

In the first test, culture supernatants were screened for the presence of antibodies to $ppp(A2'p5')_nA$ by using $I(Suc)_2$-A2′p5′A tyrosine methyl ester [^{125}I]iodo $(Suc)_2$ A2′p5′A tyrosine methyl ester as the labeled probe: 72 of 141 supernatants were positive. A second test was devised to select the hybrids that should be cloned. We measured the inhibition of binding of the labeled probe by the following compounds at two different concentrations: $(A2'p5')_2A$; $ppp(A2'p5')_2A$; A3′p5′A3′p5′A; ATP. Hybrids giving the best affinity and specificity were cloned by micromanipulation, and 13 resulting clones were expanded. Two of them, IIC3 and IIIB4, were grown to high density before the supernatants were harvested.

Determination of Ig Classes. This was performed by immunodiffusion assays. The antibodies were tested against the following immunosera: anti-μ, anti-δ, anti-(γ1 + γ2), anti-γ2a and anti-γ2b (kindly provided by H. Bazin), anti-γ2c, and anti-α.

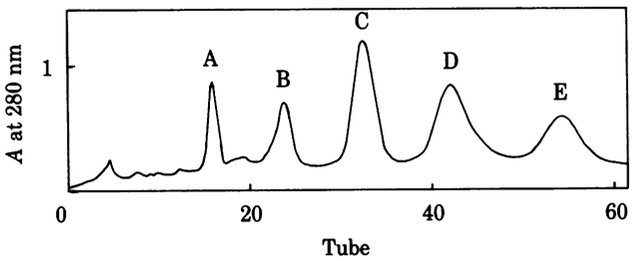

FIG. 1. Purification of Suc-A2′p5′A derivatives. A2′p5′A was dissolved in distilled water (10 mg in 1 ml) and alkalinized with 400 μl of 4 M KOH at 4°C; this solution was immediately mixed with 60 mg of lyophilized succinic anhydride and allowed to stand 5 min at room temperature. A trace of [^{14}C]succinic anhydride was added to permit location of the excess succinic acid. The medium was loaded on a AG 1 ×2 column (formate form) (1.5 × 27 cm) equilibrated in distilled water. Elution was performed with a pH gradient (250 ml of 0.05 M HCOOH to 250 ml of 1 M HCOOH). Volume per tube, 7 ml. Five peaks were obtained: A, 10%; B, 10%; C, 30%; D, 30%; E, 20%. Peak A clearly corresponded to unreacted A2′p5′A. The excess succinic acid was located between peaks B and C.

Binding Measurements. One hundred microliters of antibody (dilution, 1:60,000) in 0.1 M sodium citrate at pH 6.2 containing bovine serum albumin at 1 mg/ml was mixed with 50 μl of $I(Suc)_2$A2′p5′A tyrosine methyl ester (200,000 cpm/ml) in 0.1 M sodium citrate at pH 6.2 containing bovine serum albumin at 2 mg/ml and 50 μl of citrate buffer or A2′p5′A derivatives in citrate buffer. After a 22-hr incubation at 8°C, 100 μl of human plasma and 300 μl of polyethylene glycol 6,000 (25% in water) were added. The mixture was vortexed and left for 5 min at −20°C. A 10-min centrifugation at 2,000 rpm pelleted the protein. The ratio B/T was calculated. Blank values (i.e., radioactivity precipitated without antibodies) were always <5%.

RESULTS

Synthesis, Purification, and Identification of Suc-A2′p5′A Derivatives. Esterification of A2′p5′A by succinic anhydride was performed in alkaline medium. The presence of four differently reactive hydroxyl groups on A2′p5′A generated a complex set of succinylated isomers. Resolution of the succinylation mixture into five peaks (A–E) on anion exchange columns (AG 1 ×2 and μBondapak) indicated an increasing number of neg-

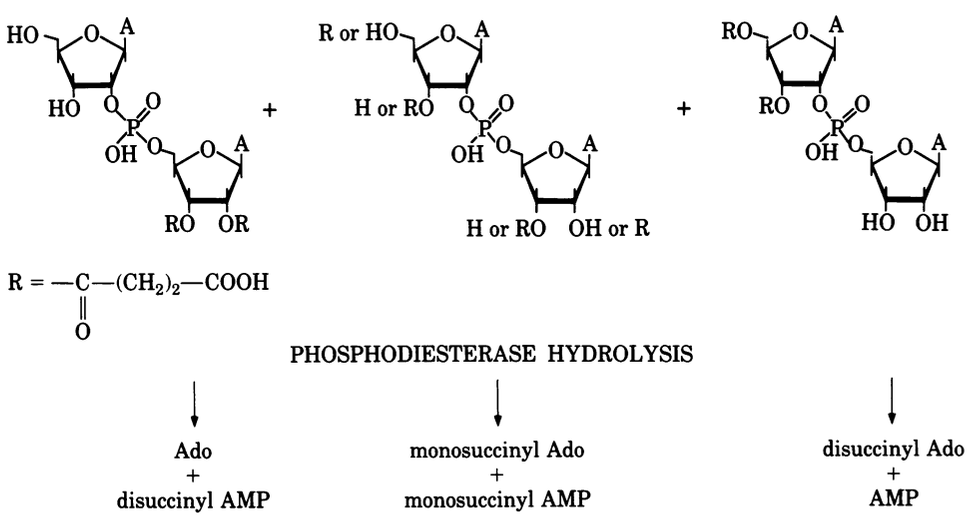

FIG. 2. Phosphodiesterase hydrolysis pattern of $(Suc)_2$A2′p5′A. Center structure represents the isomer in which each ribose is monosuccinylated on one of its two possible positions.

ative charges due to the increasing number of Suc groups on A2'p5'A up to four (Fig. 1). For all of them, UV spectra were similar to the spectrum for A2'p5'A, indicating that adenine had not been altered. Mild alkaline hydrolysis which reverted the carboxylic ester bonds, gave the original A2'p5'A; prolonged incubation with 1 M KOH gave adenosine and 2' and 3' AMP.

Phosphodiesterase hydrolysis was used to locate the position of the Suc groups both by the rate of hydrolysis and by the composition of the resulting products. Their separation was achieved by HPLC. Each product was identified by its number of negative charges and quantified. Fig. 2 shows as an example the phosphodiesterase hydrolysis pattern of $(Suc)_2A2'p5'A$. Table 1 summarizes the interpretation of the data obtained after HPLC analysis of the products formed by phosphodiesterase hydrolysis of each peak. Hydrolysis of peak A to 50% adenosine and 50% AMP confirmed peak A as A2'p5'A. Peak B was rapidly and completely hydrolyzed to four products, of which two were monosuccinylated. It can be identified as monosuccinyl Suc-A2'p5'A (78% with Suc on the 2'- or 3'-OH of the second ribose and 22% with Suc on the 5'- or 3'-OH of the first ribose). Hydrolysis of peak C was quick and complete and gave all the products predicted in Fig. 2. The percentages allowed the conclusion that peak C $(Suc)_2A2'p5'A$ (80% with the two succinyl groups on the 2'- and 3'-OH of the second ribose and 20% with a Suc link on the first ribose and the other one on the second and a negligible amount with the two Suc on the 5'- and 3'-OH of the first ribose). Products of hydrolysis of peak D were either mono- or disuccinylated. Therefore, peak D is $(Suc)_3A2'p5'A$ (60% with a Suc on the first ribose and two on the second and 30% with two Suc on the first ribose and one on the second). Peak E is clearly $(Suc)_4A2'p5'A$.

Suc-A2'p5'A and $(Suc)_2A2'p5'A$ were rapidly and completely hydrolyzed by phosphodiesterase but a part of the $(Suc)_3$ and $(Suc)_4$ derivatives still remained even after 90-min hydrolysis. The esterification of the 3'-OH of the first ribose by a succinyl molecule should hinder the binding of the phosphodiesterase. These data also show the preferential esterification of the 2'- and 3'-OH of the second ribose because hydrolysis of mono-, di- and trisuccinyl derivatives gave Suc-AMP as the major product.

Because $(Suc)_2A2'p5'A$ was the major component formed and was 80% succinylated on the 2'- and 3'-OH of the second ribose, the most distant positions from the diester bond, it was used in the synthesis of the immunogen and labeled antigen.

Synthesis of Immunogen and ^{125}I-Labeled A2'p5'A. $(Suc)_2$-A2'p5'A was coupled to human serum albumin by 1-ethyl-3,3' dimethylisopropylcarbodiimide (2 mol/mol) for use as an immunogen.

$(Suc)_2A2'p5'A$ was coupled to tyrosine methyl ester by using ethyl chloroformate as described (20). The reaction mixture was chromatographed under the same conditions as the succinylation mixture (Fig. 3). In addition to tyrosine methyl ester, two main products were formed which were nitrosonaphthol positive. They were almost pure in fractions C1 and C2 which should be the mono- and dityrosilated derivatives of $(Suc)_2A2'p5'A$ according to their positions on the chromatogram and their A_{260}/A_{280} ratios. Further purification was achieved by separation of the products after labeling with ^{125}I.

Table 1. Products of phosphodiesterase hydrolysis of peaks A, B, C, D, E*

	Product found, %				
Product	Peak A	Peak B	Peak C	Peak D	Peak E
Adenosine	50	39	40	—	—
Suc-A	—	11	10	30	—
$(Suc)_2A$	—	—	—	15	50
AMP	50	11	—	—	—
Suc-AMP	—	39	10	15	—
$(Suc)_2AMP$	—	—	40	30	50

* Peak A, A2'p5'A; peak B, Suc-A2'p5'A; peak C, $(Suc)_2A2'p5'A$; peak D, $(Suc)_3A2'p5'A$; peak E, $(Suc)_4A2'p5'A$.

Aliquots of products C1 and C2 were iodinated by chloramine-T as described (20) and the resulting products were purified on a Sephadex G-25 (superfine) column with 0.1 M citrate at pH 6.2. Except for the excess of iodide, C1 gave two main products (C1.1, C1.2) and C2 gave three (Fig. 4). For each of them, we tested the binding to several dilutions of antiserum and the displacement of the binding by A2'p5'A. With C1.2, 90% was bound to an excess of antibody and it was fully displaceable by increasing concentrations of A2'p5'A. Therefore it was used as the labeled analog.

Specificity of the Monoclonal Antibodies. From the hybrids selected for their capacity to bind [^{125}I]iodo$(Suc)_2A2'p5'A$ tyrosine methyl ester and for their specificity toward A2'p5'A, 13 clones were obtained. The 13 monoclonal antibodies were analyzed by inhibition of binding of the labeled probe with 11 different compounds: A2'p5'A, $(A2'p5')_2A$, $(A2'p5')_3A$, $(A2'p5')_4A$, pppA2'p5'A, ppp$(A2'p5')_2A$, ppp$(A2'p5')_3A$, ppp$(A2'p5')_4A$, $(A3'p5')_2A$ ATP, and adenosine.

Their properties were compared to those of antiserum. The crossreactivity factor was expressed as the ratio concentration of ligand/concentration of A2'p5'A at $B/T = \frac{1}{2}(B/T)_0$ (Table 2). All the monoclonal antibodies and the antiserum displayed the same pattern of specificity whatever the ligand was. They did not distinguish between dephosphorylated tri-, tetra-, and pentamers; the presence of the triphosphate on the 2-5A molecule affected the binding, dramatically for the dimer but less and less for the higher polymers. The monoclonal antibodies as well as the antiserum did distinguish the 2'-5' diester bond from the 3'-5' diester bond; they recognized ATP and adenosine poorly.

Clones IIC_3 and $IIIB_4$ were expanded and the resulting monoclonal antibodies plus the antiserum were investigated. Reference curves were drawn with Suc-A2'p5'A and $(Suc)_2$-A2'p5'A. Antibody concentration and dissociation constants (K_d) were calculated from Scatchard analysis. Electrofocusing was performed and IgG classes were determined.

As expected, the affinity of these antibodies was maximal for $(Suc)_2A2'p5'A$ which was used in the immunogen ($K_d = 8 \times 10^{-12}$ M for antiserum, 6.6×10^{-12} M for IIC_3, and 6.4×10^{-12} M for $IIIB_4$); it was slightly lower for Suc-A2'p5'A. Crossreactivity factors were the same for monoclonal antibodies and antiserum: 0.33 for $(Suc)_2A2'p5'A$ and 0.40 for Suc-A2'p5'A.

The electrofocusing patterns revealed that the antiserum contained only two populations of antibodies and that the one with the more acidic pI was secreted by IIC_3 and $IIIB_4$ clones.

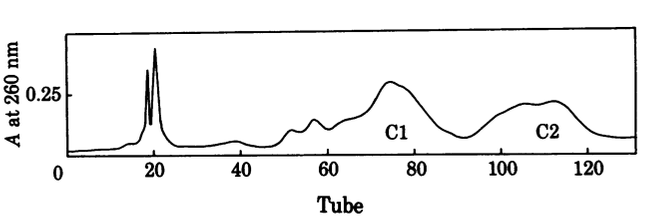

FIG. 3. Purification of $(Suc)_2A2'p5'A$ tyrosine methyl ester with the same chromatographic system as in Fig. 1. Volume per tube, 3.7 ml. C1 and C2 had UV spectra that were the combination of those of A2'p5'A and tyrosine methyl ester. They were nitrosonaphthol positive and they migrated on Polygram (EtOH/0.1 M CH_3COONH_4, (5:2) with $R_F = 0.50$ and 0.39 for C1 and C2, respectively.

Table 2. Crossreactivity of antiserum and monoclonal antibodies to ppp(A2'p5')$_n$A

Antiserum	Crossreactivity factor*							
	(A2'p5')$_n$A†	pppA2'p5'A	ppp(A2'p5')$_2$A	ppp(A2'p5')$_3$A	ppp(A2'p5')$_4$A	(A3'p5')$_2$A	Adenosine, $\times 10^{-6}$	ATP, $\times 10^{-6}$
	2.8	11,000	1,071	758	300	8,500	6.0	6.6
IC_4	3.3	10,000	1,300	1,000	200	14,000	4.7	7.6
ID_4	2.7	10,000	1,000	800	480	12,000	4.4	7.0
IIC_3	2.5	11,000	1,100	790	310	12,000	4.4	6.7
IIB_2	3.5	12,000	1,300	1,200	290	19,000	5.4	9.2
IIB_4	3.1	10,000	1,300	1,100	310	13,000	5.3	6.1
$IIIB_4$	2.6	10,000	1,250	790	350	10,000	5.9	9.1
$IIID_1$	3.1	11,000	1,200	1,000	270	17,000	5.2	7.3
$IIID_3$	3.2	13,000	1,400	950	250	14,000	4.0	6.0
ID_2	3.0	12,000	1,000	950	200	14,000	5.1	5.8
IIB_5	3.2	11,000	1,200	800	290	13,000	4.9	5.0
VA_3	3.0	10,000	1,000	910	550	14,000	5.1	7.2
VA_5	2.5	13,000	1,000	910	280	15,000	4.0	6.6
VIA_3	4.4	10,000	1,400	940	340	14,000	6.4	6.5

* Crossreactivity factor is the ratio concentration of ligand/concentration of A2'p5' at $B/T = \frac{1}{2}(B/T)_0$.
† $n = 2$–4.

These immunoglobulins are IgG2b, and their concentrations in the culture medium were 412 and 30.6 nM for IIC_3 and $IIIB_4$, respectively, compared to 2.85 nM for the antiserum.

Contribution of the Different Parts of (A2'p5')$_n$A to the Binding of the Monoclonal Antibodies: Thermodynamic Analysis. When the antigen presents several possible binding sites for the same antibody, as is the case for (A2'p5')$_n$A when $n > 1$, the affinity constant K_a ($= 1/K_d$) is the sum of the individual constants K for the different sites taken separately as long as the polymer is small enough to have only one site occupied at any one time by the antibody:

$$K_a = \frac{(\text{complex 1}) + (\text{complex 2}) + \dots}{(\text{free antigen}) \times (\text{free antibody})} = K_1 + K_2 + \dots .$$

There are six different kinds of binding sites, corresponding to the six possible neighborhoods of the A2'p5'A unit. With each one is associated an individual constant: K_0 for A2'p5'A; K_1 for A2'p5'A2'p; K_2 for ppp5'A2'p5'A; K_3 for ppp5'A2'p5'A2'p; K_4 for p5'A2'p5'A; and K_5 for p5'A2'p5'A2'p. These can be calculated from the experimental data on the affinity of the antibody for the different oligomers according to the following equations (with experimental value for antibody $IIIB_4$):

$$(\text{A2'p5'})_2\text{A}\,\frac{K_1 + K_4}{K_0} = 1/2.6 \quad [1]$$

$$(\text{A2'p5'})_3\text{A}\,\frac{K_1 + K_5 + K_4}{K_0} = 1/2.6 \quad [2]$$

$$(\text{A2'p5'})_4\text{A}\,\frac{K_1 + 2K_5 + K_4}{K_0} = 1/2.6 \quad [3]$$

$$\text{ppp5'A2'p5'A}\,\frac{K_2}{K_0} = 1/10^4 \quad [4]$$

$$\text{ppp5'(A2'p5')}_2\text{A}\,\frac{K_3 + K_4}{K_0} = 1/1{,}250 \quad [5]$$

$$\text{ppp5'(A2'p5')}_3\text{A}\,\frac{K_3 + K_5 + K_4}{K_0} = 1/780 \quad [6]$$

$$\text{ppp5'(A2'p5')}_4\text{A}\,\frac{K_3 + 2K_5 + K_4}{K_0} = 1/350. \quad [7]$$

Eq. 7 is redundant with Eq. 6 and probably less precise because of possible double antibody binding. Comparison of Eqs. 2 and 3 to Eq. 1 give K_5 negligible with respect to $K_1 + K_4$. Complete resolution was achieved by introducing an additional assumption $K_3/K_2 = K_5/K_4$ which means that 2-O phosphorylation has similar thermodynamical effects on 5'-mono- and 5'-triphosphorylated A2'p5'A. Final results are listed in Table 3. They show that the neighborhood of the A2'p5'A unit strongly influences the binding of antibody. The free 5'-OH A2'p5'A2'OH form is the best ligand, not far from the succinylated forms. Then comes 5' OH A2'p5'A2'p which is recognized almost

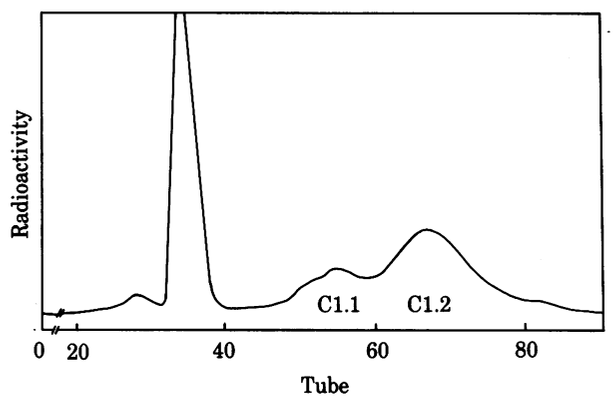

FIG. 4. Purification of [^{125}I]iodo(Suc)$_2$A2'p5'A tyrosine methyl ester. C1 (2.5 nmol) was iodinated by 1.5 mCi of [^{125}I]iodide in the presence of 20 μg of chloramine-T in 50 μl of 0.1 M phosphate buffer at pH 7.5. The reaction was stopped by sodium metabisulfite and the mixture was loaded on Sephadex G-25 (superfine) column (0.9 × 50 cm) and eluted with 0.1 M citrate at pH 6.2. The major peak was identified as iodide in excess. C1.2 was used as a labeled probe because 90% was bound to an excess of antibodies and fully displaceable by A2'p5'A.

Table 3. Relative affinity of monoclonal antibody $IIIB_4$ for the different binding sites present in ppp(A2'p5')$_n$A oligonucleotides

Compound	Relative affinity
A2'p5'A	1/1
A2'p5'A2'p---	1/2.6
---p5'A2'p5'A	1/1,370
---p5'A2'p5'A2'p---	1/2,120
ppp5'A2'p5'A	1/10,000
ppp5'A2'p5'A2'p---	1/15,900

equally well. The presence of a phosphate, and more so for a triphosphate, on the 5'-OH has a drastic inhibitory effect. Therefore, antibodies are essentially bound on the 5'-OH end of the dephosphorylated polymers (2-5A core) whereas they are bound primarily on the 2'-OH end and secondarily on the central A2'p5'A units of the triphosphorylated polymers.

DISCUSSION

"2-5A oligonucleotides" is the generic name of a family of oligomers of adenosine linked by 2'-5' phosphodiester bonds. They were found in cells in two forms—either triphosphorylated [ppp(A2'p5')$_n$A] or dephosphorylated [(A2'p5')$_n$A] (12). The role of ppp(A2'p5')$_n$A ($2 < n < 4$) as an intracellular mediator of the antiviral effect of interferon is clearly established, and more general implications relative to cell growth and differentiation are suspected (3). The exact biological significance of the dephosphorylated compounds is still unknown. To gain further insight in this complex system, we developed monoclonal antibodies against these molecules. Because they are constituted of a recurring unit, it is hopeless to develop antibodies specific for each oligomer. On the contrary, it is feasible to raise monoclonal antibodies against the repetitive unit that make possible the quantitative determination of all the oligomers with the same sensitivity. For this purpose, A2'p5'A was selected as unique antigenic determinant. It was coupled to the carrier via succinyl links attached on the 2'- and 3'-OH of the second ribose moiety in order to keep the particular spatial conformation of the 2'-5' phosphodiester bond intact.

Such an immunogen induced antiserum of restricted heterogeneity as we have already observed in similar cases (20), because 2 days before fusion the antiserum was diclonal, showing a linear Scatchard plot with (Suc)$_2$A2'p5'A. The affinity, $1.3 \times 10^{11}\ M^{-1}$, is the highest we ever obtained for mononucleotide haptens. The contribution of the succinyl link to the affinity is weak (3-fold) compared to mononucleotides (20- to 200-fold for cyclic GMP and cyclic AMP, up to 2,600-fold for cyclic CMP). This illustrates the negative correlation that exists between the size of the hapten and the increase of affinity due to the link (21). The phosphodiester bond in 3'-5' instead of 2'-5' is poorly recognized, as are adenosine and ATP, clearly showing that this antiserum was directed against the peculiar conformation due to the 2'-5' bond (22).

We did not detect monoclonal antibodies of higher specificity and affinity than the antiserum probably because one clone was already largely dominant in the animal. Thus, the 13 monoclonal antibodies share the properties of the antiserum. Thermodynamic analysis has shown that the 5'-OH free extremity is the predominant binding site of the dephosphorylated isomers whatever their length. On the contrary, triphosphorylated isomers are bound by their secondary sites: the 2'-OH end and, to a lesser extent, central A2'p5'A units so that an increase of the length results in an increase of apparent affinity. Simple phosphatase treatment restores the high-affinity site 5'-OH A2'p5'A and the independence of affinity from the size of the polymers.

Together with an ^{125}I-labeled 2-5A analog, these antibodies allow a radioimmunoassay of the 2-5A oligonucleotides: without phosphatase treatment, dephosphorylated compounds are quantified on a true molar basis (in terms of number of oligomers and not in terms of number of subunits); after phosphatase treatment, 5'-phosphorylated isomers are quantified as well. They are also suitable for intracellular localization of 2-5 A by immunocytochemistry. These analytical tools should be useful to study the dynamics of 2-5A in processes in which it is already implicated and to investigate its potential role in other biological systems.

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