Phosphorothioate Analogues of (2′–5′)$(A)_4$: Agonist and Antagonist Activities in Intact Cells†

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ABSTRACT: Metabolically stable phosphorothioate tetramer analogues of (2′–5′)$(A)_n$ with R_p and/or S_p chirality in the 2′–5′-phosphodiester linkages constitute a new class of antiviral agents since they mimic the effects of interferons. Three of the diastereomeric 5′-monophosphates (i.e., pRpRpRp, pSpRpRp, and pRpSpSp) bind to and activate RNase L from extracts of HeLa cells. However, the pSpSpSp (2′–5′)-$(A)_4$-phosphorothioate is unique in that it binds to, but cannot activate, RNase L to cleave rRNA. When microinjected into the cytoplasm of HeLa cells followed by virus infection, the pRpRpRp, pSpRpRp, and pRpSpSp (2′–5′)$(A)_4$-phosphorothioates demonstrate antiviral activity, as does (2′–5′)$(A)_4$ox-red, an active (2′–5′)$(A)_n$ analogue. When microinjected simultaneously with (2′–5′)$(A)_n$ox-red, the pSpSpSp (2′–5′)$(A)_4$-phosphorothioate inhibits activation of RNase L in HeLa cells, thereby blocking direct protection of vesicular stomatitis virus. The agonist and antagonist properties of pRpRpRp and pSpSpSp, respectively, are transient probably as a consequence of the hydrolysis of the 5′-monophosphate and formation of the less active (2′–5′)$(A)_4$-phosphorothioate cores. The possible use of these (2′–5′)$(A)_4$-phosphorothioates as tools for dissecting the biological significance of the (2′–5′)$(A)_n$ system or in antiviral chemotherapy is discussed.

It is well established that the (2′–5′)$(A)_n$ synthetase/RNase L system is important in the antiviral mechanism of external stimuli, e.g., interferon and dsRNA [for review, see Lengyel (1982)]. In addition, this system may be important in the regulation of cell growth (Wells & Mallucci, 1985). The (2′–5′)$(A)_n$ synthetase converts ATP to 2′–5′ oligoadenylates, (2′–5′)$(A)_n$.[1] (2′–5′)$(A)_n$ exerts its biological effect by binding to and activating its only known target enzyme, the unique endoribonuclease, RNase L [see Johnston and Torrence (1984) for a review]. Agonists and antagonists of RNase L thus appear as possible tools for a better understanding of the biological relevance of the (2′–5′)$(A)_n$ system as well as for antiviral and antineoplastic chemotherapy. Since enzymatic degradation of (2′–5′)$(A)_n$, which has been shown to be very fast in cell-free extracts, most probably limits its biological activity in intact cells as well, a plethora of structurally modified (2′–5′)$(A)_n$ molecules have been reported with the goal of characterizing the binding and activation processes of RNase L. These analogues have revealed either increased or decreased biological activity. On the other hand, a 5′-methylphosphorothioate derivative of (2′–5′)$(A)_n$ was observed to be an interesting inhibitor of RNase L in mouse L929 cells (Watling et al., 1985). A β,γ-difluoromethylene derivative also acts as an antagonist of RNase L in HeLa cells (Bisbal et al., 1987); however, this β,γ-difluoromethylene derivative has been synthesized enzymatically from β,γ-difluoromethylene ATP with a very low yield.

Recently, we have reported on the synthesis and biological properties of stereochemically modified (2′–5′)$(A)_n$ molecules in which R_p and S_p chirality has been introduced into the (2′–5′)$(A)_n$ backbone (Kariko et al., 1987a,b; Suhadolnik et al., 1987). The R_p and S_p phosphorothioate analogues of nucleotides, oligonucleotides, RNA, and DNA are excellent biochemical probes for numerous nucleotide-requiring enzymes (Eckstein et al., 1982; Eckstein, 1985; Kuchta et al., 1988). Phosphorothioate substitution has produced a class of enzymatically and chemically synthesized analogues with favorable stereochemical, physicochemical, and biological properties [for review, see Eckstein and Gish (1989)]. In particular, the phosphorothioate analogues of (2′–5′)$(A)_n$ made it possible to differentiate the stereochemical requirements for the binding and activation processes of RNase L. Furthermore, of the four diastereomers of (2′–5′)$(A)_3$-phosphorothioate core, the SpSp core is the most stable to nuclease digestion (Kariko et al., 1987a). The SpSp trimer core and its 5′-monophosphate interfere with the activation of RNase L in that *they can bind*

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[1] Abbreviations: (2′–5′)$(A)_n$, a series of oligomers of adenylic acid with (2′–5′)-phosphodiester linkages and a triphosphate at the 5′ end, with n usually >2; MEM, minimal essential medium; HPLC, high-performance liquid chromatography; RNase, ribonuclease; pfu, plaque-forming unit(s); VSV, vesicular stomatitis virus; moi, multiplicity of infection; RpRpRp, RpSpSp, SpRpRp, and SpSpSp tetramer cores, phosphorothioate analogues of (2′–5′)$(A)_4$ cores with R_p and S_p stereoconfigurations in the three chiral centers with assignment of configuration from the 5′ terminus to the 2′/3′ terminus; pRpRpRp, pRpSpSp, pSpRpRp, and pSpSpSp, 5′-monophosphorylated tetramer phosphorothioate analogues of (2′–5′)$(A)_4$ monophosphate. Enzymes: T4 RNA ligase (EC 6.5.1.3); T4 polynucleotide kinase (EC 2.7.1.78); (2′–5′)-oligoadenylate synthetase (EC 2.7.7.19); (2′–5′)$(A)_n$-dependent endoribonuclease or RNase L (EC 3.1.2.7); (2′–5′)-phosphodiesterase (EC 3.1.1.3).

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to but cannot activate RNase L, as determined by the lack of hydrolysis of poly(U)–3′[^{32}P]pCp and by the inability to cleave ribosomal RNA to specific cleavage products (Kariko et al., 1987b). A similar observation was obtained by using the calcium phosphate coprecipitation technique; the pRpRpRp tetramer but not the pSpSpSp tetramer inhibits protein synthesis in L929 cells (Suhadolnik et al., 1989).

To more clearly understand the implications of these observations, it now becomes essential to ascertain the specific interactions occurring in the nucleotide binding domain of RNase L and/or other (2′–5′)(A)$_n$ binding proteins. We have approached this goal by the direct microinjection of the R_p and S_p (2′–5′)(A)$_4$-phosphorothioates into the cytoplasm of HeLa cells followed by the infection with vesicular stomatitis virus (VSV). The microinjection of nucleotides, double-stranded RNA, and proteins is an established technology for transmembrane passage of normally impermeable molecules (Higashi & Sokawa, 1982; Bayard et al., 1985; Silhol et al., 1986; Defilippi et al., 1986).

In this paper we establish that the direct microinjection of microquantities of the R_p and S_p (2′–5′)(A)$_4$-phosphorothioates into the cytoplasm of HeLa cells results in either the inhibition of the antiviral response or the antagonism of the RNase L system, whereby virus replication is not inhibited.

Materials and Methods

Materials. Media for cell culture were obtained from Eurobio and sera from Flow Laboratories. T4 RNA ligase was purchased from Pharmacia and T4 polynucleotide kinase from Bethesda Research Laboratories. [^{32}P]pCp (specific activity 3000 Ci/mmol) and [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) were purchased from Amersham.

Cell Culture and Viruses. L929 cells were grown in minimal essential medium supplemented with 5% (v/v) donor horse serum, 3 g/L bactotryptose phosphate broth, 3.4 g/L glucose, 60 IU/mL penicillin, and 50 μg/mL streptomycin; HL929 (an RNase L deficient subclone of mouse L929; Carter et al., 1987) was grown in Dulbecco's modified Eagle medium supplemented with 5% (v/v) calf serum. HeLa cells were maintained in RPMI 1640 medium supplemented by 5% (v/v) fetal calf serum and antibiotics as above. Vesicular stomatitis virus (VSV) (Indiana strain) was grown in L929 cell monolayers and titrated by plaque assay.

Synthesis and Characterization of (2′–5′)(A)$_4$ox-red. Enzymatically polymerized (2′–5′)(A)$_4$ oligomers were modified at the 3′-terminal ribose residue to increase their stability with regard to phosphodiesterase degradation, as previously described (Bayard et al., 1984). The purity of the modified oligomers was assessed by HPLC on a μBondapak C_{18} column in 50 mM ammonium phosphate buffer, pH 7.2, according to the method of Brown et al. (1981).

Synthesis of (2′–5′)(A)$_4$-Phosphorothioates. The four (2′–5′)(A)$_4$-phosphorothioate core diastereoisomers were chemically synthesized by the phosphotriester approach and separated by preparative thin-layer chromatography on silica gel as described for the (2′–5′)-phosphorothioate trimer cores (Kariko et al., 1987a).

5′-Monophosphorylation of (2′–5′)(A)$_4$-Phosphorothioate Cores with T4 Polynucleotide Kinase. The 5′-monophosphates of the (2′–5′)(A)$_4$-phosphorothioate cores were synthesized from ATP or [γ-^{32}P]ATP with T4 polynucleotide kinase as described for the (2′–5′)(A)$_3$-phosphorothioate cores (Kariko et al., 1987b), except that the total reaction volume was 100 μL. Progress of the reaction was monitored by reverse-phase HPLC with a Waters Associates radial compression system (Z module) and a μBondapak C_{18} Radial Pak cartridge (8 mm × 10 cm). For separation, 50 mM ammonium phosphate, pH 7.0 (buffer A), and methanol/H_2O (1:1 v/v) (solvent B) were used in a linear gradient (t = 1 min, 0% solvent B; t = 31 min, 70% solvent B) at a flow rate of 2 mL/min.

Radiobinding Assay for (2′–5′)(A)$_4$ Analogues. Radiobinding assays were performed according to the method of Knight et al. (1980), using an S10 unfractionated HeLa cell extract as the source of (2′–5′)(A)$_n$-dependent endoribonuclease (RNase L). The labeled probe (2′–5′)(A)$_4$pCp was synthesized by ligation of [^{32}P]pCp (specific activity 3000 Ci/mmol) on (2′–5′)(A)$_4$ with T4 RNA ligase (Silverman et al., 1981).

Radiocovalent Affinity Labeling of RNase L. The covalent labeling procedure of Wreschner et al. (1982) was utilized with some modifications. After removal of the terminal phosphate of (2′–5′)(A)$_4$-3′[^{32}P]pCp, the 3′-ribose residue was quantitatively oxidized with freshly prepared sodium metaperiodate (10 mM final concentration, pH 4.75) for 1 h at 4 °C. The oxidized mixture was then incubated with cell extracts in the presence of cyanoborohydride (20 mM final concentration, pH 8) for 20 min at 4 °C and for a further 20 min at 22 °C. The incubated preparation was then analyzed by 10% SDS/PAGE (Bisbal et al., 1989).

Core–Cellulose Purification of HeLa Cell RNase L. RNase L was purified from extracts of IFN-treated HeLa cells utilizing (2′–5′)(A)$_4$-core–cellulose as described by Silverman (1985) with the following exception. The pellet containing RNase L bound to core–cellulose was suspended in an equal volume of rRNA cleavage assay reaction buffer [5 mM Tris–HCl, pH 7.6, 1.25% (v/v) glycerol, 20 mM KCl, 1.25 mM $Mg(OAc)_2$]. The core–cellulose-bound RNase L was used as the source of RNase L in rRNA cleavage assays as described below.

Ribosomal RNA Cleavage Assays. rRNA cleavage assays were performed according to the procedure described by Wreschner et al. (1981) with the following modification. Extracts of HL929 cells were prepared as described (Carter et al., 1987) and incubated for 1 h at 30 °C with or without (2′–5′)(A)$_4$ or (2′–5′)(A)$_4$-phosphorothioate in the absence or presence of core–cellulose-bound HeLa cell RNase L prepared as described above. The total RNA was extracted, denatured, and analyzed by electrophoresis on 1.8% (v/v) agarose gels. The gels were stained with ethidium bromide, and the RNA bands were visualized under ultraviolet light.

Cell Microinjection. HeLa cells were grown on small pieces of glass (2 mm^2) at densities that allowed about 200 cells to become attached to each glass fragment as previously described (Graessman & Graessman, 1983). Injections were monitored under a Leitz Diavert phase-contrast microscope with a magnification of 320. An average volume of 0.5 pL (i.e., approximately $^1/_{10}$ of the cell volume) was injected in the cytoplasm of each cell with 0.5-μm-diameter glass micropipets.

Assay of Antiviral Activity. Cells were infected at the indicated times (30 min after microinjection) with VSV at a multiplicity of 10 for 1 h at 37 °C in RPMI medium supplemented with 5% (v/v) fetal calf serum. Unabsorbed viruses were carefully removed by three washings with RPMI medium containing 10% (v/v) fetal calf serum. The viruses produced were titrated 18 h later by an end-point assay as described previously (Milhaud et al., 1983).

Thin-Layer Chromatographic Analysis of (2′–5′)(A)$_4$-Phosphorothioates and Their Degradation Product. [^{32}P]-(2′–5′)(A)$_4$, [^{32}P]pRpRpRp, and [^{32}P]pSpSpSp and their degradation products were separated on cellulose (Eastman Chromagram, 13254) thin-layer chromatography plates de-

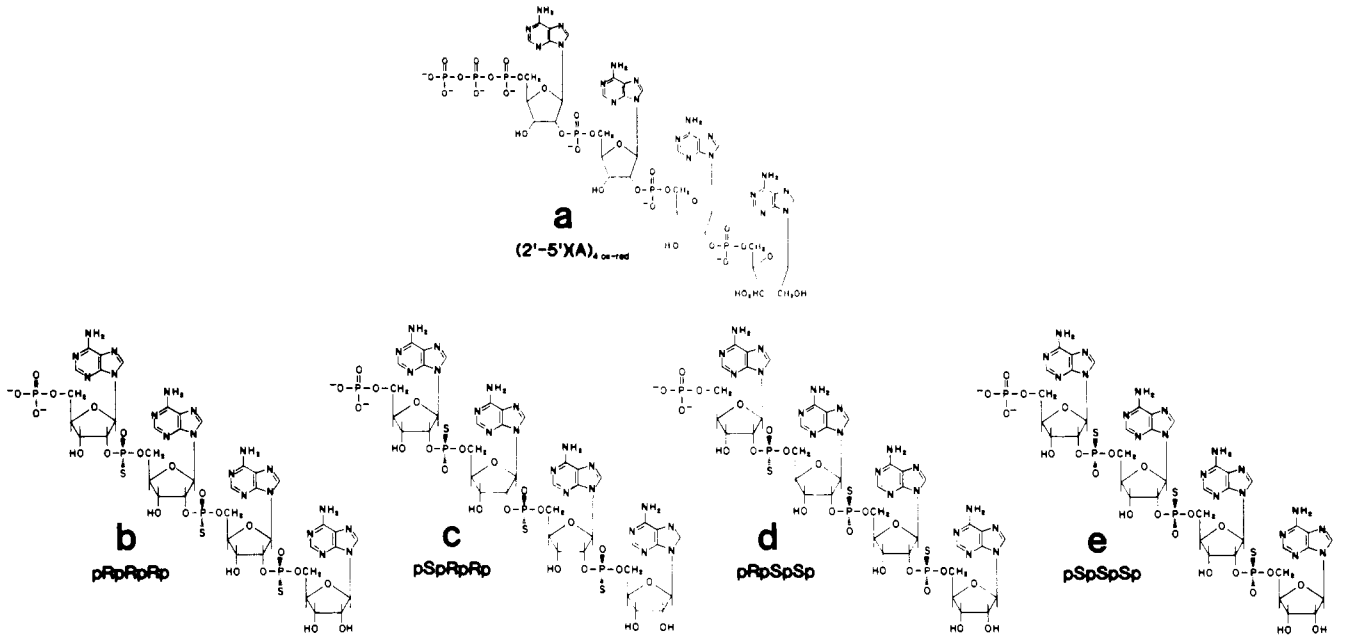

FIGURE 1: Structure of (2′–5′)(A)$_4$ox-red and of the (2′–5′)(A)$_4$-phosphorothioate analogues. (a) (2′–5′)(A)$_4$ox-red; (b) pRpRpRp; (c) pSpRpRp; (d) pRpSpSp; (e) pSpSpSp.

Table I: Transfer of γ Phosphate from ATP by T4 Polynucleotide Kinase to the 5′-Hydroxyl of 3′,5′- and 2′,5′-Oligoadenylates

oligonucleotide	% transfer[a]
(2′–5′)(A)$_4$	37.4
RpRpRp tetramer core	15.2
SpRpRp tetramer core	27.5
RpSpSp tetramer core	33.5
SpSpSp tetramer core	29.7
(3′–5′)(A)$_4$	>99

[a] Percent conversion based on calculated areas under the HPLC curve for each oligonucleotide core and corresponding 5′-monophosphate. Percent yields are an average of four separate determinations.

veloped with isobutyric acid/NH_4OH/H_2O (66/1/33 v/v/v). The radioactive components were located by autoradiography with Kodak X-Omat film and quantified by liquid scintillation spectrometry on a Beckman LS-100C liquid scintillation spectrometer with counting efficiency of 99% for ^{32}P.

Phosphatase Activity in HeLa Cell Extracts. Phosphatase activity was determined as described by Bayard et al. (1984). Phosphatase activity was estimated by measuring the disappearance of [^{32}P](2′–5′)(A)$_4$, [^{32}P]pRpRpRp, or [^{32}P]pSpSpSp and the concomitant appearance of liberated $^{32}P_i$ after TLC as described above.

RESULTS

5′-Monophosphorylation of (2′–5′)(A)$_4$-Phosphorothioate Cores with T4 Polynucleotide Kinase. The 5′-monophosphates of the (2′–5′)(A)$_4$-phosphorothioate cores were synthesized from ATP with T4 polynucleotide kinase. The formation of the 5′-monophosphates was determined by HPLC analysis. Subsequent hydrolysis to cores was accomplished with 5′-nucleotidase. The phosphorylation of the four (2′–5′)(A)$_4$-phosphorothioate cores was not as dependent on stereoconfiguration as had been reported for the (2′–5′)(A)$_3$-phosphorothioate cores (Kariko et al., 1987a). The yield of the pRpRpRp was 15.2% as compared with a yield of 2.5% for the corresponding pRpRp (Table I) (Kariko et al., 1987a). The structures of the analogues are illustrated in Figure 1.

Binding of (2′–5′)(A)$_4$ox-red (2′–5′)(A)$_4$-Phosphorothioate to RNase L. The radiobinding assay described by Knight et al. (1980) is based on the ability of (2′–5′)(A)$_4$ analogues to compete with a [^{32}P](2′–5′)(A)$_4$pCp probe for specific binding to RNase L in an unfractionated cell-free extract prepared in this case from HeLa cells. As illustrated in Figure 2, there are no significant differences in the affinity of (2′–5′)-(A)$_4$ox-red and the phosphorothioate analogues for RNase L in HeLa cell extracts. As previously defined by Imai et al. (1982), the results can be expressed in terms of IC_{50}, i.e., the concentrations required to inhibit 50% of the binding of the [^{32}P](2′–5′)(A)$_4$pCp probe. Under our experimental conditions the four analogues have IC_{50} values between 2.6×10^{-9} and 5×10^{-9} M (Figure 2), the same affinity for RNase L as (2′–5′)(A)$_4$ and (2′–5′)(A)$_4$ox-red ($IC_{50} = 5 \times 10^{-9}$ M). Similar data concerning the relative affinity of these (2′–5′)(A)$_4$-phosphorothioate analogues for their target enzyme have been obtained by using the covalent binding assay initially described by Wreschner et al. (1982) (data not shown). This confirms and extends these and previous (Sobol et al., unpublished results) studies in demonstrating competitive binding by (2′–5′)(A)$_4$-phosphorothioate for the 80 kDa polypeptide described as the major (2′–5′)(A)$_n$ binding element in most

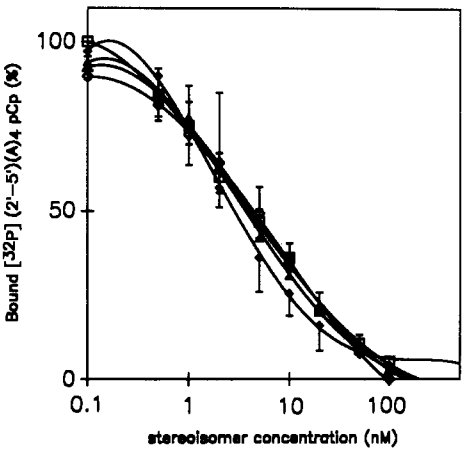

FIGURE 2: Binding of (2′–5′)(A)$_4$ox-red and its phosphorothioate analogues to RNase L. (2′–5′)(A)$_4$ox-red (■), pRpRpRp (○), pRpSpSp (△), and pSpSpSp (▲) were compared for their ability to inhibit the binding of the [^{32}P](2′–5′)(A)$_4$pCp probe to RNase L in the radiobinding assay.

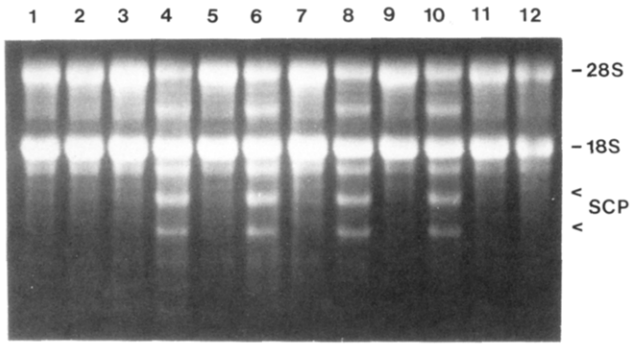

FIGURE 3: Ribosomal RNA cleavage assay with (2′–5′)(A)$_4$-phosphorothioate. Incubations were for 1 h at 30 °C; extraction and electrophoretic analysis of the RNA were as described under Materials and Methods. HL929 cell extracts were incubated in the absence (lanes 1 and 2) or presence of (2′–5′)(A)$_4$ at 10^{-9} M (lanes 3 and 4), pRpRpRp at 10^{-7} M (lanes 5 and 6), pSpRpRp at 10^{-7} M (lanes 7 and 8), pRpSpSp at 10^{-7} M (lanes 9 and 10), or pSpSpSp at 10^{-6} M (lanes 11 and 12) and core–cellulose-purified RNase L from extracts of IFN-treated HeLa cells (lanes 2, 4, 6, 8, 10, and 12). The positions of 28S and 18S rRNA are shown; the arrows indicate the positions of specific cleavage products (SCP).

cell species (Silverman et al., 1988; Bisbal et al., 1989).

Activation of RNase L in Extracts of Interferon-Treated HeLa Cells by (2′–5′)(A)$_4$-Phosphorothioates. Activation of RNase L by the (2′–5′)(A)$_4$-phosphorothioates was measured by rRNA cleavage assay using core–cellulose-purified RNase L. The pRpRpRp, pSpRpRp, and pRpSpSp activated RNase L to cleave 28S and 18S rRNA to specific cleavage products at 1×10^{-7} M (Figure 3, lanes 6, 8, and 10). However, the pSpSpSp (1×10^{-6} M) did not activate RNase L (Figure 3, lane 12). This lack of hydrolysis is consistent with the inability of the pSpSpSp to activate RNase L in intact cells, after microinjection (vide infra).

Effect of (2′–5′)(A)$_4$-Phosphorothioates on Cell Growth. Fifty HeLa cells were microinjected on day one with the indicated concentrations of (2′–5′)(A)$_4$ox-red or analogues and counted every day (Figure 4). pSpRpRp (panel B) and pRpSpSp (panel C) analogues did not modify significantly the growth properties of HeLa cells in this range of concentrations. The two analogues pRpRpRp (panel A) and pSpSpSp (panel D) slowed the growth of HeLa cells during the first 24 h when injected at intracytoplasmic concentrations of 10^{-6} M. No significant effect has been observed at lower concentrations, that is, in the range of concentrations required to exhibit an antiviral activity for pRpRpRp (vide infra). This growth inhibitory activity of (2′–5′)(A)$_4$-phosphorothioate analogues at high concentration is not accompanied by cell death (not shown).

Antiviral Activity of (2′–5′)(A)$_4$ox-red and Its Analogues in Intact Cells. The four phosphorothioate analogues and (2′–5′)(A)$_4$ox-red were compared in dose–response experiments for their capacity to inhibit the multiplication of vesicular stomatitis virus (VSV) inoculated at a moi of 10 into HeLa cells 20 min after microinjection. As illustrated in Table II the (2′–5′)(A)$_4$-phosphorothioates exhibit very different antiviral activities in this assay. The pRpRpRp analogue has an antiviral activity comparable to the activities in this assay. The pRpRpRp analogue has an antiviral activity comparable to the activity of (2′–5′)(A)$_4$ox-red, with a threshold value around 10^{-9} M. The pSpRpRp and pRpSpSp analogues have lower but significant antiviral activities, while the pSpSpSp analogue does not significantly alter virus multiplication at doses as high as 10^{-6} M.

Antagonism of RNase L Activation by the pSpSpSp Analogue. The properties of the pSpSpSp analogue, i.e., its normal affinity for RNase L, its lack of cleavage of rRNA, and its absence of antiviral activity, led us to expect it to be a new

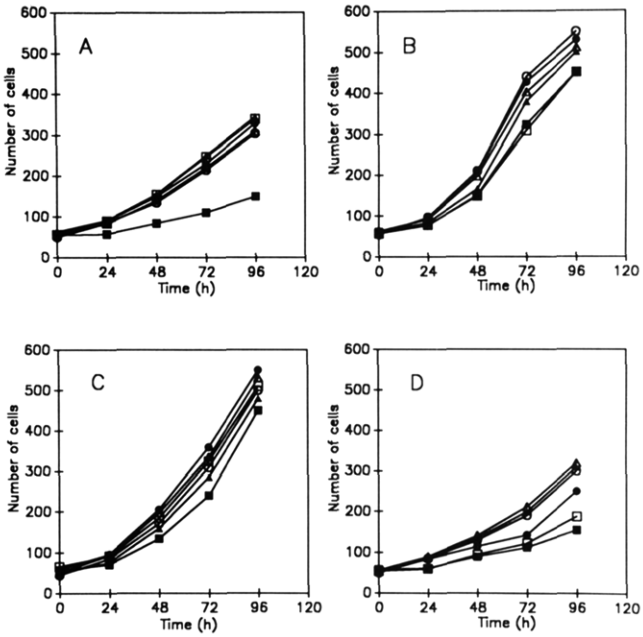

FIGURE 4: Growth curve of cells microinjected by the different (2′–5′)(A)$_4$-phosphorothioate analogues. 50 HeLa cells were injected at time 0 with the (2′–5′)(A)$_4$-phosphorothioate analogues pRpRpRp (A), pSpRpRp (B), pRpSpSp (C), and pSpSpSp (D) at the indicated concentrations: (■) 10^{-6} M; (□) 10^{-7} M; (▲) 10^{-8} M; (△) 10^{-9} M; and (●) H_2O. The number of cells was determined every day and compared with a normal cellular population (○).

Table II: Antiviral Activity of Microinjected (2′–5′)(A)$_4$ox-red and Phosphorothioate Analogues against VSV[a]

treatment	intracytoplasmic concn of oligomers (M/L)	virus yield (pfu/200 cells)	antiviral act. (%)
H_2O		3.5×10^5	0
(2′–5′)(A)$_4$ox-red	10^{-9}	5.9×10^5	0
	10^{-8}	1.3×10^5	62
	10^{-7}	3.9×10^4	89
	10^{-6}	9.5×10^3	97
H_2O		6.4×10^5	0
pRpRpRp	10^{-9}	3.8×10^5	40
	10^{-8}	2.4×10^5	61
	10^{-7}	1.4×10^5	77
	10^{-6}	5.3×10^4	91
H_2O		2.1×10^5	0
pSpRpRp	10^{-9}	1.9×10^5	10
	10^{-8}	9.1×10^4	59
	10^{-7}	7.4×10^4	68
	10^{-6}	5×10^4	78
H_2O		3.3×10^5	0
	10^{-9}	3×10^5	8
	10^{-8}	2.2×10^5	32
	10^{-7}	9.1×10^4	73
	10^{-6}	8.8×10^4	73
H_2O		3.6×10^5	0
pSpSpSp	10^{-9}	3.5×10^5	1
	10^{-8}	3.1×10^5	9
	10^{-7}	3×10^5	17
	10^{-6}	4×10^5	0

[a] 200 HeLa cells were microinjected with 0.5 pL each of (2–5′)-(A)$_4$ox-red or phosphorothioate analogue at the indicated concentrations; 30 min after microinjection, cells were challenged with VSV at a moi of 10, and the virus titers were determined 18 h later by an end-point assay in L929 cells (Milhaud et al., 1983). Each concentration curve has been established in a separate experiment with its internal control constituted by sterile water microinjected cells.

Table III: Antiviral Activity of (2′–5′)(A)$_4$ox-red and pSpSpSp Analogue Microinjected Together[a]

treatment	intracytoplasmic concn of oligomers (M/L)	virus yield (pfu/200 cells)	antiviral act. (%)
H_2O		1.9×10^5	0
(2′–5′)(A)$_4$ox-red	5×10^{-7}		
+pSpSpSp	5×10^{-10}	9.4×10^3	95
	5×10^{-9}	4.6×10^4	78
	5×10^{-8}	6.4×10^4	68
	5×10^{-7}	1.6×10^5	24
H_2O		1.2×10^5	0
(2′–5′)(A)$_4$ox-red	5×10^{-8}		
+pSpSpSp	5×10^{-10}	2.5×10^4	81
	5×10^{-9}	5.6×10^4	57
	5×10^{-8}	7.7×10^4	49
	5×10^{-7}	1.2×10^5	3

[a] 200 HeLa cells were microinjected with 0.5 pL each of (2′–5′)-(A)$_4$ox-red at the concentration of 5×10^{-7} or 5×10^{-8} M together with pSpSpSp analogue at the indicated concentrations; 30 min after the microinjection, cells were challenged with VSV at a moi of 10, and the virus titers were determined 18 h later as described in Table II.

analogue inhibitor of RNase L. To demonstrate such inhibitory activity, the pSpSpSp analogue was microinjected together with (2′–5′)(A)$_4$ox-red in HeLa cells and the cells were challenged with VSV as described above. Under these conditions a dose-dependent inhibition of the antiviral activity of (2′–5′)(A)$_4$ox-red was observed (Table III). These results strongly suggest that the pSpSpSp analogue is an effective inhibitor of RNase L activation in HeLa cells.

Stability of the Activity of the Analogues in Intact Cells. The (2′–5′)(A)$_4$-phosphorothioates are stable against phosphodiesterase in cell-free extracts (Kariko et al., 1987a). The stability of their activity (antiviral activity for the pRpRpRp analogue and antagonist activity for the pSpSpSp analogue) was monitored in intact cells after microinjection. The pRpRpRp analogue was microinjected at a final concentration of 10^{-6} M, and the cells were challenged with VSV at different times after microinjection. Although its antiviral activity decreases with time, the pRpRpRp analogue exhibits good functional stability in intact cells, since it still retains 50% maximal antiviral activity when injected 18 h before virus challenge (Figure 5A). The antagonist activity of the pSpSpSp analogue is less stable in these experimental conditions. Indeed, the ability of the pSpSpSp analogue to inhibit the activation of RNase L by (2′–5′)(A)$_4$ox-red rapidly decreases when the delay between agonist and antagonist microinjection is increased. The antagonist activity completely disappears if the time interval between the two injections is greater than 4 h (Figure 5B). This might be due to the dephosphorylation of (2′–5′)(A)$_4$-phosphorothioate monophosphates to the corresponding cores whose affinity for RNase L has been shown to be 100 times lower (Suhadolnik et al., 1989). Indeed, the half-life of the 5′-phosphate moiety of pRpRpRp and pSpSpSp is about 2 h in HeLa cell extracts in the absence of ATP (Figure 6A); no appreciable dephosphorylation could be observed, however, when 1 mM ATP and an ATP-regenerating system were added to the extract (Figure 6B).

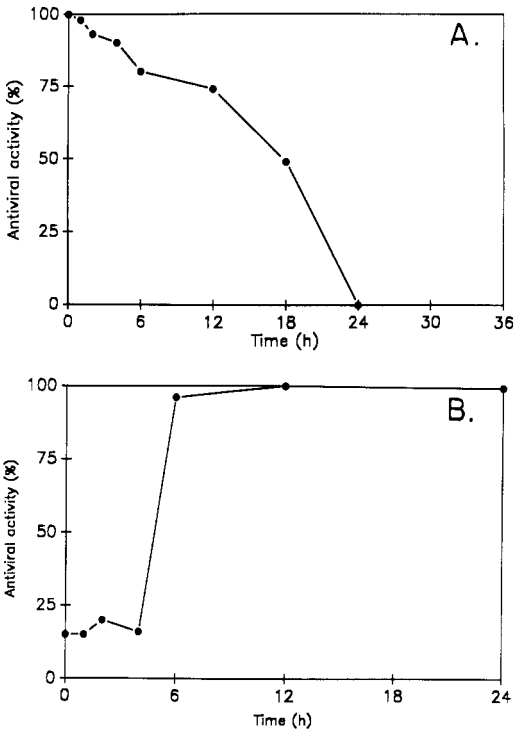

FIGURE 5: (A) Stability of the antiviral activity of pRpRpRp analogue. 200 HeLa cells were microinjected with pRpRpRp at the intracytoplasmic concentration of 10^{-6} M. The cells were then challenged with VSV (moi of 10) at various times after microinjection as indicated. Virus yields were determined 18 h later as described in Table II. The antiviral activity (percent) is relative to the activity of pRpRpRp 30 min after its microinjection (defined as 100%). (B) Stability of the antagonist activity of pSpSpSp analogue. 200 HeLa cells were microinjected with the pSpSpSp analogue at the intracytoplasmic concentration of 5×10^{-7} M, and (2′–5′)(A)$_4$ox-red (5×10^{-7} M) was microinjected at the different times indicated after the first microinjection. Thirty minutes after the second microinjection, cells were challenged with VSV (moi of 10); virus yields were determined 18 h later as described in Table II. The antiviral activity (percent) is relative to the activity of (2′–5′)(A)$_4$ox-red microinjected alone (defined as 100%).

Discussion

Introducing R_p and S_p chirality into the (2′–5′)-phosphodiester bonds of the (2′–5′)(A)$_n$ molecule has revealed several new aspects of the stereodynamics involved in the binding and activation processes of RNase L by this new class of biological response modifiers. The pRpRpRp phosphorothioate reduces VSV multiplication when microinjected into HeLa cells to the same extent as the (2′–5′)(A)$_4$ox-red molecule, a known agonist of RNase L (Bayard et al., 1984). This result extends our earlier in vitro observation that the pRpRpRp binds to and activates RNase L to degrade 18S and 28S rRNA equally as well as (2′–5′)(A)$_3$ (Kariko et al., 1987b; Suhadolnik et al., 1989). Examination of the stereochemical importance of the placement of R_p and S_p chirality in the (2′–5′)(A)$_4$-phosphorothioate analogues of (2′–5′)(A)$_4$ reveals an interference with the antiviral process in that the antiviral activity is lower, but significant, when the R_p chiral center at the 5′ terminus of the pRpRpRp molecule is changed to an S_p chiral center, i.e., the pSpRpRp molecule. Similarly, modification of R_p chirality to S_p chirality at the middle and the (2′/3′)-terminal chiral center, i.e., pRpSpSp, also lowers the antiviral activity. The pSpSpSp analogue inhibits activation of RNase L when injected simultaneously with (2′–5′)(A)$_4$ox-red into HeLa cells challenged with VSV, thus providing a new class of RNase L antagonist. The minimal structural requirements for antiviral activity are not met by either the (2′–5′)(A)$_3$-phosphorothioate pSpSp (not shown) or the (2′–5′)(A)$_4$-phosphorothioate pSpSpSp.

The replacement of the nonbridging oxygens of the phosphates in the (2′–5′)-phosphodiester bonds of the (2′–5′)(A)$_4$ molecule with sulfur introduces the properties of chirality and increased metabolic stability at the internucleotidic linkages. The observed discrimination between R_p and S_p diastereoi-

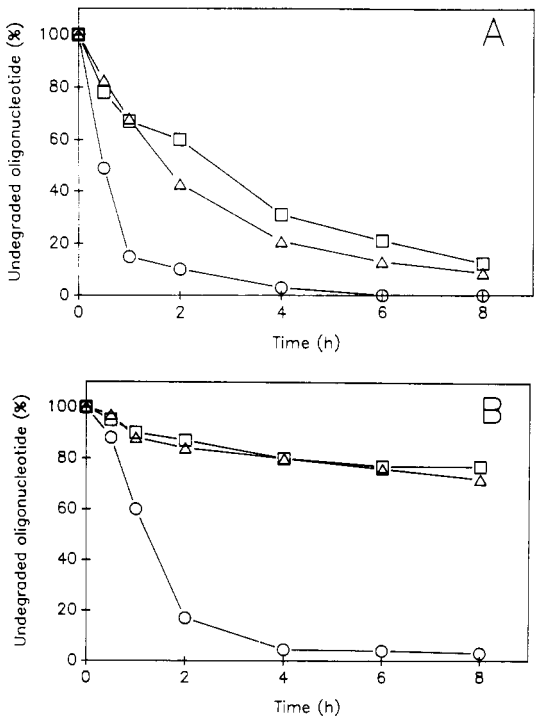

FIGURE 6: Kinetics of degradation of (2′–5′)(A)$_4$ and (2′–5′)(A)$_4$-phosphorothioate. [^{32}P](2′–5′)(A)$_4$ (○), [^{32}P]pRpRpRp (□), and [^{32}P]pSpSpSp (△) were incubated with HeLa cell extracts in the absence (A) or presence (B) of 1 mM ATP and an ATP-regenerating system (see Materials and Methods). Incubations were stopped at the indicated times by heating (100 °C, 2 min), and denatured proteins were removed by centrifugation (10000g, 2 min). Residual oligonucleotides in the supernatant were analyzed by thin-layer chromatography as described under Materials and Methods.

somers shown in cell-free experiments (Kariko et al., 1987a,b) is reflected in their biological activities in intact cells. This type of stereochemical discrimination is reminiscent of the studies by Stec et al. (1982), with the R_p and S_p isomers of cyclic AMP phosphorothioates. Neither the agonists nor the antagonist analogue described here shows a long-lasting activity in intact cells. It may be that dephosphorylation of the 5′-phosphate by cellular phosphatases limits their efficacy in intact cells. Our cell-free studies indeed indicate a dephosphorylation which could be prevented by the addition of an ATP-regenerating system. Whether or not these cell-free studies can be extrapolated to the intact cell remains, however, to be established because a 5′-monophosphorothioate is not hydrolyzed by phosphatases.

Agonist and antagonist data as shown here for (2′–5′)-(A)$_4$-phosphorothioates have also been reported for trimers (Suhadolnik et al., 1989), although in less detail. Our purpose in studying tetramers is to permit the conjugation of these agonists and antagonists to polypeptide carriers as effective vectors for delivery to intact cells via the periodate oxidation of the (2′/3′)-ribosyl moiety while leaving the active trimer moiety intact (Bayard et al., 1986).

Supplementary Material Available

Chemical synthesis of the four phosphorothioate tetramer cores and HPLC analysis of the 5′-monophosphate of SpSpSp tetramer core by T4 polynucleotide kinase (4 pages). Ordering information is given on any current masthead page.

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Differential Scanning Calorimetry of the Unfolding of Myosin Subfragment 1, Subfragment 2, and Heavy Meromyosin†

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ABSTRACT: The thermal unfolding of rabbit skeletal heavy meromyosin (HMM), myosin subfragment 1, and subfragment 2 has been studied by differential scanning calorimetry (DSC). Two distinct endotherms are observed in the DSC scan of heavy meromyosin. The first endotherm, with a T_m of 41 °C at pH 7.9 in 0.1 M KCl, is assigned to the unfolding of the subfragment 2 domain of HMM based on scans of isolated subfragment 2. The unfolding of the subfragment 2 domain is reversible both in the isolated form and in HMM. The unfolding of subfragment 2 in HMM can be fit as a single two-state transition with a ΔH_{vh} and ΔH_{cal} of 161 kcal/mol, indicating that subfragment 2 exists as a single domain in HMM. The unfolding of subfragment 2 is characterized by an extraordinarily large ΔC_p of approximately 30 000 cal/(deg·mol). In the presence of nucleotides, the high-temperature HMM endotherm with a T_m of 48 °C shifts to higher temperature, indicating that this peak corresponds to the unfolding of the subfragment 1 domain. This assignment has been confirmed by comparison with isolated subfragment 1. The stabilizing effect of AMPPNP was significantly greater than that of ADP. The vanadate-trapped ADP species was slightly more stable than M·AMPPNP with a T_m at 58 °C. The unfolding of subfragment 1, both in the isolated form and in HMM, was irreversible. Only a single endotherm was noted in the DSC scans of the subfragment 1 domain of HMM and in freshly prepared subfragment 1 complexes. There is no evidence in the DSC data for preferential stabilization of any subdomain of the head by the binding of nucleotides.

Scanning calorimetry provides an experimental demonstration of cooperative domain structure and domain interactions in proteins (Privalov, 1979; Sturtevant, 1987). The term "domain" has been used in various ways to describe structural units in a folded protein (Privalov, 1982; Zehfus, 1987; Vibert & Cohen, 1988). The most straightforward, objective, and experimentally testable definition of a domain is an independent, cooperative unit. A domain may affect the stability of neighboring domains, but their folded structure is independent. According to this definition, domains can be experimentally demonstrated by observing the unfolding reactions of proteins.

Protein unfolding can be followed by a number of techniques: e.g., by monitoring changes in circular dichroism, fluorescence, NMR spectra, and proteolytic rates. Analysis of proteolysis data can be complicated since the proteolysis itself may influence the unfolding process. Spectroscopic probes can be used to determine the equilibrium constant of the unfolding reaction as a function of temperature, from which an apparent van't Hoff enthalpy can be derived if the process is reversible (Privalov, 1979, 1982). Differential scanning calorimetry (DSC) provides not only an indication of the progress of the unfolding reaction (and therefore a van't Hoff enthalpy) but also a direct measure of the enthalpy of the process (a calorimetric enthalpy) (Privalov, 1979). The calorimetric and van't Hoff enthalpies are equal for single-domain proteins (Privalov, 1979; Sturtevant, 1987). For proteins with multiple domains, either there will be multiple peaks in the DSC scan or, if the peaks overlap, the calorimetric enthalpy will exceed the van't Hoff enthalpy.

Myosin is an oligomeric protein of molecular weight 530 000 (Harrington & Rodgers, 1984). It is composed of two heavy chains and four light chains. The amino-terminal residues of the heavy chain fold to form a globular head, and the remainder of the molecule is involved in dimerization to form an extended coiled-coil segment. It is the globular heads which contain the ATPase and actin binding sites. The globular heads can be isolated in homogeneous form following limited proteolysis of myosin with α-chymotrypsin in the presence of EDTA (Weeds & Taylor, 1975; Margossian & Lowey, 1982). The isolated heads are referred to as subfragment 1. Proteolysis of myosin with α-chymotrypsin in the presence of excess divalent cations yields heavy meromyosin and light meromyosin (Weeds & Pope, 1977). Heavy meromyosin is a water-soluble two-headed fragment lacking a large part of the helical rod. The largely helical portion of heavy meromyosin (HMM)[1] linking the two heads together is referred to as subfragment 2, and may be obtained by treating HMM

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[1] Abbreviations: DTE, dithioerythritol; S-1, myosin subfragment 1; S-2, myosin subfragment 2; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; HEPPS, *N*-(2-hydroxyethyl)piperazine-*N'*-3-propanesulfonic acid; HMM, heavy meromyosin; T_m, midpoint temperature of a transition; TRIS, tris(hydroxymethyl)aminomethane.

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