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A systemic antiviral resistance-inducing protein isolated from *Clerodendrum inerme* Gaertn. is a polynucleotide:adenosine glycosidase (ribosome-inactivating protein)

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Abstract Two systemic antiviral resistance-inducing proteins, CIP-29 and CIP-34, isolated from *Clerodendrum inerme* Gaertn. leaves, were tested for ribosome-inactivating properties. It was found that CIP-29 has the characteristics of a polynucleotide: adenosine glycosidase (ribosome-inactivating protein), in that it inhibits protein synthesis both in cell-free systems and, at higher concentrations, in cells, and releases adenine from ribosomes, RNA, poly(A) and DNA. As compared with other known RIPs, CIP-29 deadenylates DNA at a high rate, and induces systemic antiviral resistance in susceptible plants.

Key words: Ribosome-inactivating protein; Polynucleotide:adenosine glycosidase; Antiviral protein

1. Introduction

It is well known that extracts from many plants possess antiviral activity, some of them exerting their activity locally, i.e. when applied at the site of infection, whilst others induce a systemic resistance to viruses (for a recent review see [1]). In some cases the antiviral activity, but not the systemic resistance induction, was due to proteins which were provisionally named ribosome-inactivating proteins (RIPs, review in [2]). Some proteins with antiviral activity were indeed found to be RIPs [3] and, conversely, the antiviral activity in plant extracts led to the identification of some new RIPs [4]. Recently two basic glycoproteins, called CIP-29 and CIP-34, which induced a very high degree of systemic resistance against tobacco mosaic virus in *Nicotiana tabacum* cv. Samsun NN, were purified from *Clerodendrum inerme* (Verbenaceae) [5]. CIP-29 was found to share several characteristics with RIPs, viz. (i) it is a monomer with a molecular mass of 29 kDa, (ii) it has a basic isoelectric point, and (iii) it is fairly resistant to temperature and proteolytic digestion. However, it did not cross-react with sera against other RIPs, namely dianthin, momordin and saporin [5], as expected, since RIPs from plants belonging to different families usually do not cross-react [6].

These apparent similarities prompted us to investigate the two proteins in greater detail. One of them, CIP-29, showed the characteristics of a RIP with polynucleotide:adenosine glycosidase activity, and its enzymatic activity was studied on several polynucleotides which had previously been found to be substrates of RIPs (see Section 4 below).

2. Materials and methods

2.1. Chemicals

The antiviral proteins CIP-29 and CIP-34 were purified from leaves of *C. inerme* as described earlier [5]. [^{14}C]L-Leucine was from Amersham International, Buckinghamshire, UK. Adenine and tRNA were from Sigma Chemical Co., St. Louis, MO, USA. Poly(A) and rRNA from *Escherichia coli* (16S+23S, m.w. 1.75×10^6) were from Boehringer GmbH, Mannheim, Germany. DNA from herring sperm (Sigma) was mechanically sheared and made RNA-free by treatment with DNase-free RNase A (Boehringer) for 2.5 h at 37°C. DNA was then repeatedly precipitated in ethanol to remove the enzyme. Standard adenine was from Sigma. All other chemicals were as in previous work [7].

2.2. Cell cultures

The cell lines used, namely mouse 3T3 (fibroblasts), and human HeLa (carcinoma), NB 100 (neuroblastoma) and BeWo (chorioncarcinoma) were maintained as monolayer cultures in RPMI 1640 medium supplemented with antibiotics and 10% fetal calf serum, in a humidified atmosphere containing 5% CO_2, at 37°C. Subcultures were obtained by trypsin treatment of confluent cultures. The JM cell line (monocyte-derived) was grown in suspension and treated as described [8].

2.3. Determination of enzyme activities

The effect of CIP-29 and CIP-34 on protein synthesis in a cell-free system (a rabbit reticulocyte lysate) was studied essentially as described [7], with the details given in the legend to Fig. 1. Protein synthesis by cells was determined as in [9]. The IC_{50} (concentration giving 50% inhibition) was calculated by linear regression analysis.

Unless otherwise stated, polynucleotide:adenosine glycosidase activity was determined by measuring adenine released from various substrates by HPLC [10] essentially following the procedure of [11] as described by [12]. Reactions were run for 40 min at 30°C in a final volume of 50 μl containing 50 mM Na-acetate, pH 4.0, 100 mM KCl and RIP and substrate as indicated in the legends to the appropriate figures. Controls were run without RIP, and a standard curve of adenine was run with each experiment.

3. Results

The proteins purified from *C. inerme* inhibited protein synthesis by a rabbit reticulocyte lysate, CIP-29 with an IC_{50} of 5.48×10^{-10} M (16 ng/ml) and CIP-34 with an IC_{50} of 8.74×10^{-8} M (3 μg/ml) (Fig. 1). The low specific activity of CIP-34 led us to analyze this protein by chromatography on a Blue-Sepharose column, to which RIPs bind [2]. Most of the protein, containing approximately 6% of the inhibitory activity, was not bound to the column. The bound protein was eluted with a NaCl gradient giving several small peaks,

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Abbreviations: RIP, ribosome-inactivating protein

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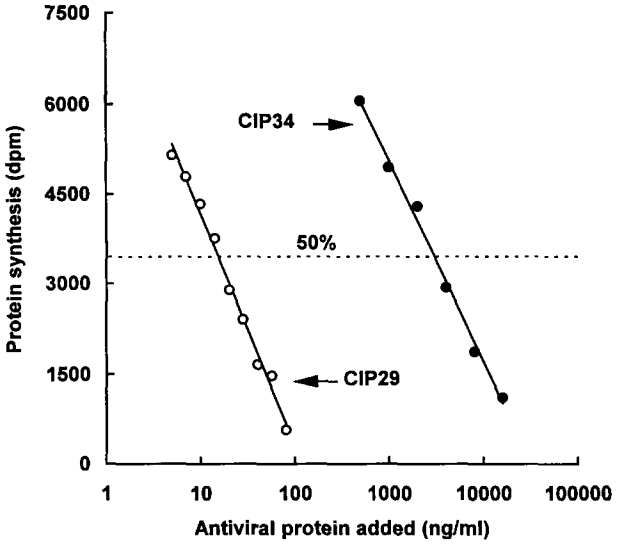

Fig. 1. Effect of CIP-29 (○) and CIP-34 (●) on cell-free protein synthesis. The effect of the proteins was assayed on globin mRNA-directed protein synthesis by a rabbit reticulocyte lysate. The reaction mixture contained, in a final volume of 62.5 μl: 10 mM Tris-HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM Mg-acetate, 1 mM ATP, 0.2 mM GTP, 15 mM phosphocreatine, 3 μg of creatine kinase, 0.05 mM amino acids (minus leucine), 89 nCi of L-[^{14}C]leucine, and 25 μl of rabbit reticulocyte lysate. Incubation was for 5 min at 28°C. Control values were 6781 dpm.

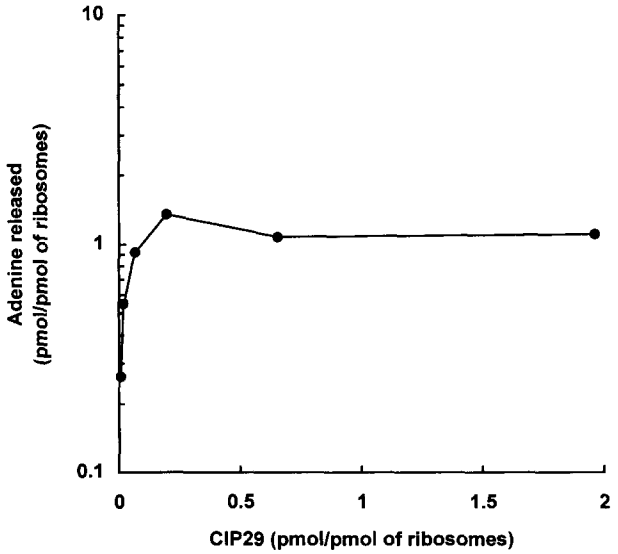

Fig. 2. Release of adenine from purified rat liver ribosomes by CIP-29. Reaction was run for 40 min at 30°C in a final volume of 50 μl containing 30 pmol of ribosomes, 20 mM Tris-HCl, pH 6.8, 100 mM NH_4Cl, 7 mM Mg-acetate, 1 mM DTT and RIPs at the indicated concentrations.

and the activity was distributed along the gradient, not coinciding with the protein peaks (results not shown). Further experiments were performed with CIP-29, on the assumption that only this could be a pure RIP (see Section 4 below). Like other type 1 RIPs, CIP-29 inhibited protein synthesis by various cell lines, although at concentrations much higher than those effective in the cell-free system (Table 1).

That CIP-29 is a RIP was confirmed by the release of adenine from rat liver ribosomes, up to 1 mol of adenine per ribosome (Fig. 2). Since at least some RIPs release adenine from purified rRNA and other polynucleotide substrates (see Section 1 above), the effect of CIP-29 was tested on different types of RNA and on DNA. It was observed that the CIP-29 released adenine from rRNA, tRNA, poly(A), as well as from DNA, the highest activity being observed with the latter substrate (Fig. 3).

4. Discussion

A characteristic of RIPs is their inhibitory effect on protein synthesis. RIPs were found to be peculiar *N*-glycosidases, and to remove a single adenine from a precise position in rRNA (A^{4324} in the case of rat liver ribosomes) (review in [13]). More recently, however, it was reported that some, but not all, RIPs remove more than one adenine residue per ribosome [14], and that saporins [15,16] and other RIPs ([12]; unpublished results by Barbieri et al.) remove adenine from RNA other than rRNA and also from DNA. Consistently, ricin removes 2′-deoxyadenine from a synthetic dodecanucleotide with a GdA-GA loop, actually at a faster rate than it removes adenine from a similar dodecanucleotide with a GAGA loop [17]. On the basis of these properties the name of polynucleotide: adenosine glycosidase was proposed for RIPs. Some RIPs, however, have been reported to cleave supercoiled ds DNA in vitro into linear forms, although they do not have any effect on linear DNA itself [18]

Of the two antiviral proteins isolated from *C. inerme* only CIP-29 showed a significant inhibitory effect on protein synthesis, both by a cell-free system and by cells, which was comparable to that of other known RIPs [2]. As observed with other RIPs [2], the effect on cells varied, BeWo and NB100 being the most sensitive, and HeLa and 3T3 the most resistant lines. That the inhibition on cells occurs only at relatively high

Table 1
Effect of CIP-29 on protein synthesis by cell lines

Cell line	Protein synthesis by controls (dpm)	IC_{50} (nM)	Correlation coefficient
HeLa	17 776 ± 1 592	> 3 330	n.d.
NB 100	7 345 ± 1 471	325 ± 183	0.96 ± 0.03
JM	6 400 ± 3 818	678 ± 196	0.99 ± 0.01
3T3	11 886 ± 5 593	> 3 330	n.d.
BeWo	53 536 ± 2 746	316 ± 81	0.99 ± 0

Cells (10^5/well, in triplicate) were incubated for 18 h in 0.5 ml of RPMI-1640 containing 10% fetal calf serum and various concentrations of CIP-29 (1.5–3330 nM). The medium was then replaced with serum- and leucine-free RPMI-1640 containing 125 nCi of L-[4,5-^{3}H]leucine and after 2 h the cells were collected and the radioactivity incorporated into protein was determined as described in [9]. Results are mean ± S.E.M. of two experiments.

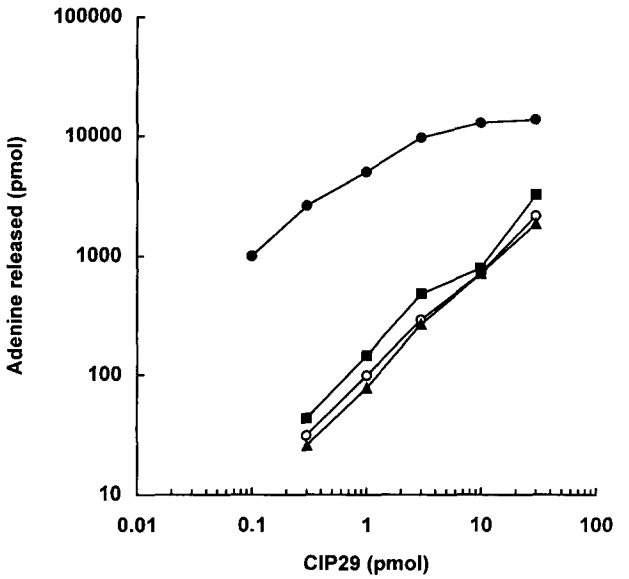

Fig. 3. Polynucleotide:adenosine glycosidase activity of the ribosome-inactivating protein CIP-29 assayed with various substrates. The substrates were herring sperm DNA (●), poly(A) (○), rRNA (▲), tRNA (□). Reactions were run for 40 min at 30°C in a final volume of 50 μl containing 20 μg of substrate, 50 mM Na-acetate, pH 4.0, 100 mM KCl and RIPs at the indicated concentrations.

concentrations suggests that the RIP internalization is not mediated by a specific receptor. The other protein, CIP-34, was shown to have an inhibitory effect at extremely high concentrations. As suspected [5], this preparation appeared to be a mixture of proteins, most of them inactive, and containing small amounts of inhibitory activities, possibly due to isoforms of the same RIP, as often occurs in plants (see for instance [9]).

In addition to inhibiting protein synthesis, CIP-29 has the enzymatic properties of RIPs in that it releases adenine from rat liver ribosomes. Like other RIPs, it is active not only on ribosomes, but also on various polynucleotides, thus being a polynucleotide:adenosine glycosidase. These effects are catalytic, as shown by molar ratio less than 1 between RIP present in the reaction mixture and adenine released. From the characteristics of their enzymatic activity, RIPs appear to be a non-homogeneous class of enzymes, in that (i) some release one, and some more than one adenine residues per ribosome [14], (ii) some are more active on poly(A) than on RNA [15,16] and (iii) have variable activity on RNA and on DNA (unpublished results). CIP-29 releases only one adenine residue from rat liver ribosomes, is not particularly active on poly(A), but is very active on DNA, more than any RIP assayed so far under the same experimental conditions. This indicates (i) that the activity of CIP-29, and presumably of other RIPs, on one substrate is independent of the activity on other substrates and (ii) that DNA appears to be the best polynucleotide substrate for CIP-29. If the effect on DNA occurs in vivo, it may contribute to the antiviral activity of CIP-29 by damaging viral or virally induced DNA.

The notion that RIPs may have a defensive role against viruses is supported by the resistance acquired by plants transfected with RIP genes [19–21] and, indirectly, by the expression of a RIP in tissues of sugar beet plants consequent to viral infection or to treatment with mediators of virally induced plant acquired resistance [22]. It should be pointed out that the exogenous application of CIP-29 rendered a susceptible plant systemically resistant to subsequent virus inoculation [5], as do RIPs which are expressed in the plant. It is possible that some other substances inducing systemic resistance to viruses (review in [1]) may also turn out to be RIPs. How a locally applied RIP can induce resistance at a distance remains unknown.

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