Enzymatic activity of toxic and non-toxic type 2 ribosome-inactivating proteins

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Abstract Ribosome-inactivating proteins (RIPs) display adenine polynucleotide glycosylase activity on different nucleic acid substrates, which at the ribosomal level is responsible for the arrest of protein synthesis. Some type 2 RIPs, namely ricin and related proteins, are extremely toxic to mammalian cells and animals whilst other type 2 RIPs (non-toxic type 2 RIPs) display three to four logs less toxicity. We studied whether a correlation exists between toxicity on cells and enzymatic activity on nucleic acids. All type 2 RIPs differ in their depurinating activity on the different substrates with differences of up to one to two logs. The toxicity of type 2 RIPs is independent of their enzymatic activity on nucleic acids or on ribosomes.

Key words: Ribosome-inactivating protein; Lectin; Adenine polynucleotide glycosylase; Protein synthesis

1. Introduction

Ribosome-inactivating proteins (RIPs) from plants are classically divided into two categories: type 1, single-chain proteins with enzymatic activity, and type 2, consisting of an enzymic A chain, similar to type 1 RIPs, linked to a B chain with lectin properties [1]. More recently a type 3 was introduced, including a single RIP from maize, in which the enzymic chain has an additional peptide tail that must be removed for the RIP to be active (review in [2]).

Type 2 RIPs, the first ones to be known, were considered to be highly toxic because their B chain binds to galactosyl-terminated receptors on the cell surface, thus allowing the entry of the A chain into the cytoplasm where it exerts its enzymatic activity on ribosomes, inhibiting cell protein synthesis with IC_{50}s in the nM range or below. Since early times it has been known that enzymatic activity was not synonymous with toxicity since *Ricinus* agglutinin (RCA 120) was scarcely toxic to cells and animals although it shares an identical A enzymatic subunit with the extremely toxic ricin [3], although this observation was considered to be an exception to the rule. Subsequently, however, a number of type 2 RIPs were identified, which have structural and enzymatic properties similar to those of ricin and related toxins but, like RCA 120, are much less toxic to cells and animals: this group of proteins was named non-toxic type 2 RIPs (reviewed in [3,4]). The reasons for this difference are not completely understood.

The enzymatic activity of RIPs was officially defined as an rRNA *N*-glycosidase (rRNA *N*-glycohydrolase, EC 3.2.2.22), after it was found that they remove an adenine residue from rRNA [5]. Subsequently some RIPs were found which removed more than one adenine per ribosome [6,7]. Furthermore, it was observed that all RIPs remove adenine not only from RNA, but also from DNA and some of them also from other adenosine-containing macromolecules [8,9], and the name polynucleotide adenine glycosylase was proposed for these proteins [10]. It is still not known whether the depurinating activity on substrates other than ribosomes has a role in the cytotoxic activity of RIPs, although precocious alterations possibly not dependent on inhibition of translation were observed in the nuclei of cells exposed to ricin or Shiga toxin [11].

From the investigations reported above, performed mainly with type 1 and with a few, toxic [8] and non-toxic [12], type 2 RIPs, it appeared that among RIPs there are differences in the enzymatic activity and in the specificity for substrates. The present investigation was undertaken to compare the enzymatic activity of a number of toxic and non-toxic type 2 RIPs on various nucleic acid substrates.

2. Materials and methods

2.1. Materials

RIPs were purified as described in the respective references: abrin a [13], cinnamomin [14], ebulin l [15], ebulin r1 and ebulin r2 [16], IRA b and IRA r [17], modeccin [18], nigrin b [19], ricin and RCA 120 [20], SNA I and SNLRP [21], mistletoe lectin I (viscumin) [22], volkensin [23]. Lectins were reduced by incubation with 1% 2-mercaptoethanol for 1 h at 37°C. Rat liver ribosomes were prepared essentially as described elsewhere [24] in RNase-free conditions. Their concentration was determined by the A_{260} as described in [25], assuming that 12.5 absorbance units/ml was equivalent to 1 mg/ml and that 1 mg contained 250 pmol of ribosomes. Ribosomes were stored in aliquots at 80°C.

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Abbreviations: RIP, ribosome-inactivating protein; hsDNA, herring sperm DNA

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Table 1
Effect of type 2 RIPs on protein synthesis by a rabbit reticulocyte lysate

RIP	Inhibition of protein synthesis (IC_{50}, nM)	
	Native protein	Reduced protein
Toxic proteins		
Abrin a	88	0.5
Ricin	84	0.1
Mistletoe lectin I	43.3	3.5
Modeccin	45	2.3
Volkensin	84	0.37
Non-toxic proteins		
RCA 120	Inactive[a]	0.05
Cinnamomin	30.5	7.4
Ebulin l	Inactive[a]	0.15
Ebulin r1	59.6	0.34
Ebulin r2	0.6	1.14
Nigrin b	>100	0.1
SNA I	2.7	1.65
SNLRP	6.03	5.74
IRA b	2.9	3.6
IRA r	5.8	4.7

Reaction mixtures contained, in a final volume of of 62.5 μl: 10 mM Tris–HCl buffer, pH 7.4, 100 mM ammonium acetate, 2 mM magnesium 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, scalar concentrations of RIP and 25 μl of rabbit reticulocyte lysate. Incubation was at 28°C for 5 min. The reaction was arrested with 1 ml of 0.1 M KOH, and two drops of H_2O_2 and 1 ml of 10% (w/v) of trichloroacetic acid were added. Precipitated proteins were collected on glass fiber discs and the radioactivity incorporated into protein was measured.
[a]At 100 nM concentration.

Herring sperm DNA (hsDNA) obtained from Sigma was mechanically sheared. *Escherichia coli* rRNA was from Boehringer Mannheim (Mannheim, Germany). Chemicals, as far as possible RNase-free, were as in previous work [9]. Water was 'milli-Q' grade (Millipore).

2.2. Enzymatic activities

Cell-free protein synthesis was assayed with a rabbit reticulocyte lysate as described in [26], and the glycosylase activity of RIPs with DNA or RNA as substrates as described in [9], details being given in the legends to the appropriate tables. The depurination of rat liver ribosomes was performed either in the same conditions used for nucleic acids, or at pH 7.8, with a reaction mixture described in the legend to Fig. 1. Adenine released was measured as described [27]. Kinetic determinations were performed with the protocol described in [28] with the aid of the program 'hyper' by J.S. Easterby or by non-linear regression analysis (XLSTAT, Addinsoft, France).

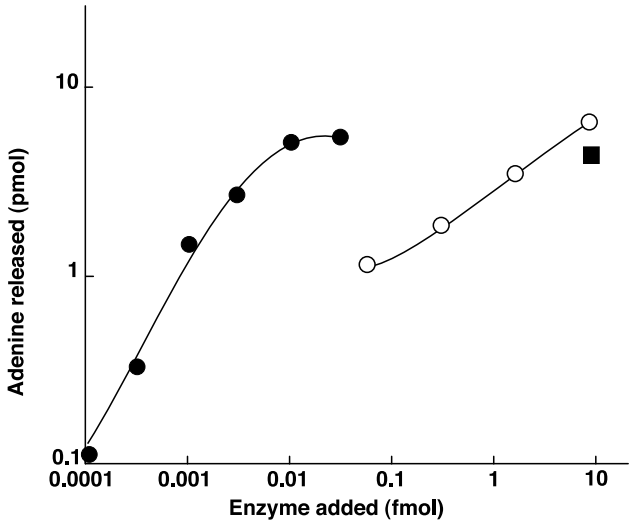

Fig. 1. Depurination of rat liver ribosomes by two toxic (● abrin a, ■ volkensin) and a non-toxic (○ RCA 120) RIPs. Reaction mixtures contained, in a final volume of 200 μl, 0.002 M Tris–HCl buffer, 0.02 M sucrose, 0.015 M KCl, 0.135 M NH_4Cl, 0.001 M Mg-acetate, 0.02 M NH_4-acetate, the appropriate amount of abrin (○) or RCA 120 and 9 pmol of ribosomes. Incubation was at 30°C for 40 min. Maximum rate was 22.3 and 0.093 pmol/min/pmol RIP for abrin a and RCA, respectively.

3. Results

The effect of type 2 RIPs on mammalian ribosomes was first evaluated in an indirect way, by assaying their inhibitory activity on protein synthesis by a rabbit reticulocyte lysate. All RIPs inhibited protein synthesis, although with very different potency (Table 1). As is known, the activity of all toxic type 2

Table 2
Glycosylase activity of RIPs on herring sperm DNA and on rRNA

RIP	Glycosylase activity (pmol adenine released/pmol RIP/min)		
Substrate	hsDNA		rRNA
	Unreduced lectin	Reduced lectin	Unreduced lectin
Toxic proteins			
Abrin a	0.67	0.03	0.63
Ricin	1.52	1.38	0.57
Mistletoe lectin I	0.28	0.38	0.24
Modeccin	0.03	0	0.22
Volkensin	0.15	0.04	0
Non-toxic proteins			
RCA 120	0.25	0.33	0.02
Cinnamomin	0.18	0.18	0.05
Ebulin l	1.38	0.18	0.06
Ebulin r1	1.51	0.44	0.13
Ebulin r2	0.94	0.07	0.05
Nigrin b	1.10	0.30	0.06
SNA I	0.05	0.04	0.06
SNLRP	0.34	0.14	0.04
IRA b	1.34	0.23	0.21
IRA r	1.79	0.51	0.23

Reaction mixtures contained, in a final volume of 50 μl: 50 mM Na-acetate buffer, pH 4.0, 100 mM KCl, 10 μg of hsDNA or rRNA and 3 pmol of RIP.

Table 3
Glycosylase activity of RIPs on rat liver ribosomes

RIP	Glycosylase activity (pmol adenine released/pmol enzyme/60 min)
Toxic proteins	
Abrin a	0.024
Ricin	0.513
Mistletoe lectin I	0.035
Modeccin	0
Volkensin	0.008
Non-toxic proteins	
RCA 120	0.083
Cinnamomin	0.014
Ebulin l	0.014
Ebulin r1	0.037
Ebulin r2	0.053
Nigrin b	0.023
SNA I	0
SNLRP	
IRA b	0.109
IRA r	0.116

Reaction mixtures contained, in a final volume of 50 μl: 50 mM Na-acetate buffer, pH 4.0, 100 mM KCl, 5 pmol of rat liver ribosomes and 10 pmol of reduced RIP (5 pmol in the case of RCA 120, consisting of a dimer with two A chains). Adenine was determined as described [27].

RIPs in the native status was relatively low, and was enhanced when they were reduced to separate their A and B chains. In the same system, the activity of five non-toxic RIPs (RCA 120, cinnamomin, ebulin l1, ebulin r1, nigrin b) was similarly low, and again was enhanced by reduction, whereas the other non-toxic lectins (ebulin r2, SNA I, SNLRP, IRA b and IRA r) already had a high activity in the native state, which was not enhanced or even slightly hampered (ebulin r2, IRA b and IRA r) by reduction.

The glycosylase activity was assayed on nucleic acids as substrates at pH 4.0, optimal for RIP activity on these substrates [29,30]. All RIPs were active on hsDNA, again within a wide range. RCA 120 was the most active, followed, in descending order, by ebulin r, ricin and abrin, whilst volkensin was the least active (Table 2). The activity was almost always decreased when the lectins were reduced. The glycosylase activity was tested also on rRNA and in all cases was lower than on hsDNA (Table 2). Under the same experimental conditions, the activity of RIPs on rat liver ribosomes was very low if not absent (Table 3), in contrast to numerous previous observations performed at pH 7, confirmed with representative RIPs (Fig. 1).

The kinetics of the glycosylase activity of ricin on hsDNA was studied, and the following values were obtained: K_m 152 μM, V_{max} 31 μmol/min, and K_{cat} 9.3 min^{-1}.

4. Discussion

The first consideration emerging from the present results is that type 2 RIPs differ considerably from each other in their enzymatic activities on various substrates. Thus the inhibitory activity on cell-free protein synthesis, an expression of damage to mammalian ribosomes, varies by more than two orders of magnitude among both toxic and non-toxic lectins, the latter being, on average, not less active than the toxic ones.

All lectins had glycosylase activity on hsDNA, but generally much less on rRNA. Again, there was no correlation with the activity on a functioning complete translation system (a rabbit reticulocyte lysate).

To be fully active on the reticulocyte lysate system, i.e. on ribosomes, most lectins must be reduced. However, two of them, ebulin r2 and IRS b, in the native state had a high activity, which was not increased by reduction. This suggests that when RIPs are in the native state, in most of them the active site does not have access to the ribosome substrate, because of steric hindrance by the B chain. It is noteworthy that the activity of all RIPs on DNA (i) in no case was increased more than 1.5-fold, and (ii) in most cases was actually significantly decreased by reduction. This indicates that the preparation of DNA utilized in the present experiments, being smaller (median length 1 kb) than ribosomes, has access to the active site of the native proteins. A ricin holotoxin containing a non-reducible covalent linker between the subunits was inactive on ribosomes, and still remained almost as toxic to mammalian cells as the native ricin [31]. This suggests that within the cells either the modified molecule undergoes some change, or the toxin could hit targets other than ribosomes.

The higher activity of RIPs at acidic than at basic pH, on both DNA and RNA [29,30], could be explained assuming that the acidic pH favors a more unlocked and rigid structure sustained by the electric repulsion of the negative polyphosphate backbone, which enables an easier accessibility of nucleic acids to RIPs. At basic pH the negative charges are neutralized, hence allowing a more compact and thus less accessible structure. Ribosomes are not depurinated under the optimal conditions for depurination of nucleic acids. These differences suggest that RIPs may act on different substrates depending upon the conditions in various cell compartments or organelles.

The activity on various substrates is not related to the toxicity of type 2 RIPs to cells and animals. The reason(s) for the different toxicity could be in the binding to cells, entry into the cytoplasm, intracellular routing and degradation of the proteins.

It should be recalled here that there are pronounced differences in sugar binding specificity between the B chains of type 2 RIP/lectins from elderberry, apparently depending on specific amino acid substitutions in the respective carbohydrate binding sites [32,33]. Basic nigrin b, a very active two-chain RIP, does not display carbohydrate binding activity and therefore has no toxicity on mice even at 40 mg/kg body weight [34]. Also, from structural data, it has been argued that ebulin l lacks the lipase active site of ricin and abrin, which has been suggested to have a role in the toxicity of ricin [35].

In a study on cell entry and intracellular fate of ricin and nigrin, one of the less toxic RIPs, it was observed that nigrin b, as compared with ricin, binds equally well to cells, but a greater proportion of it goes to the lysosomes, where it is degraded and expelled from cells [36]. Moreover, the low lysine content may render the ricin A chain resistant to proteolytic degradation [37].

In conclusion, the differences in the cytotoxicity of RIPs cannot be accounted for by their enzymatic activity. Presumably differences in their intracellular fate are important, on which, however, comparative studies are still too scarce to give sufficient information.

The differences in the activity reported here, other properties of RIPs, and their different concentrations and localiza-

tion in plant tissues [1,4,38] all together point to possible different biological roles played by type 1 and type 2 RIPs in plants. Some type 1 RIPs accumulate in leaf senescence [39] and in fruit ripening [38] whilst others are constitutive and do not change with leaf life cycle [38]. In contrast, type 2 RIPs almost disappeared in leaf senescence [40,41]. This clearly supports the belief that RIPs having a common enzymatic mechanism, namely adenine polynucleotide glycosylase, could be effector agents in different biological events of the plant.

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