The differential catalytic activity of ribosome-inactivating proteins saporin 5 and 6 is due to a single substitution at position 162

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Saporin, a type I ribosome-inactivating protein produced by the soapwort plant *Saponaria officinalis* belongs to a multigene family that encodes its several isoforms. The saporin seed isoform 6 has significantly higher N-glycosidase and cytotoxic activities compared with the seed isoform 5, although the two have identical active sites. In the present study, we have investigated the contribution of non-conservative amino acid changes outside the active sites of these isoforms towards their differential catalytic activity. The saporin 6 residues Lys^{134}, Leu^{147}, Phe^{149}, Asn^{162}, Thr^{188} and Asp^{196} were replaced by the corresponding saporin 5 residues, Gln^{134}, Ser^{147}, Ser^{149}, Asp^{162}, Ile^{188} and Asn^{196}, to generate six variants of saporin 6, K134Q, L147S, F149S, N162D, T188I and D196N. By functional characterization, we show that the change in amino acid Asn^{162} in saporin 6 to aspartic acid residue of saporin 5 contributes mainly to the lower catalytic activity of saporin 5 compared with saporin 6. The non-involvement of other non-conservative amino acids in the differential catalytic activity of these isoforms was confirmed with the help of the double mutations N162D/K134Q, N162D/L147S, N162D/F149S, N162D/T188I and N162D/D196N.

Key words: catalytic activity, ribosome-inactivating protein (RIP), ricin, saporin 5, toxin, translation.

INTRODUCTION

Plant RIPs (ribosome-inactivating proteins) are potent toxins that kill cells by inactivating ribosomes [1]. RIPs catalytically depurinate major rRNA by exerting a highly specific N-glycosidase activity that cleaves an adenine base, A4324, which forms part of a tetranucleotide 'GA^{4324}GA' sequence in a conserved loop in rat 28 S rRNA termed the SRL (sarcin-ricin loop) [2,3]. The removal of this adenine causes structural changes in the rRNA that disrupt binding of elongation factors to the ribosomes, and as a result protein synthesis is arrested at the translocation step [4]. RIPs have been divided into two categories: the type I RIPs such as saporin, pokeweed antiviral protein and tricosanthin, which consist of a single polypeptide chain; and the type II RIPs such as ricin and abrin, which contain an A chain that is essentially equivalent to a type I RIP, as well as a lectin-like B chain, that facilitates their entry into the cytosol [5].

Saporin represents a multigene family of proteins isolated in the form of more than nine different isoforms from various parts of the soapwort plant *Saponaria officinalis* [6]. The first recombinant clone of saporin was obtained from the cDNA library from leaves of the soapwort plant [7]. The various isoforms of saporin differ from each other in both physicochemical as well as biological properties. The saporin isoforms have been designated by the source tissue and peak number in which they were obtained during ion-exchange chromatography of crude tissue extracts [8]. Saporin 6 constituted the major peak, peak 6, of the preparation from seeds, accounting for approx. 0.4 % of the whole seed weight or 7 % of the total seed protein [6].

A functional comparison of two saporin seed isoforms, saporin 5 and saporin 6 showed saporin 6 to have a significantly higher catalytic and cytotoxic activity compared with saporin 5 [8,9]. Although the active site residues of saporin, Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179} and Trp^{208}, are conserved in both the isoforms, the two isoforms differ from each other at 12 positions (Table 1) [10]. Of these, the amino acid residues Lys^{134}, Leu^{147}, Phe^{149}, Asn^{162}, Thr^{188} and Asp^{196} of saporin 6 that are replaced by Gln^{134}, Ser^{147}, Ser^{149}, Asp^{162}, Ile^{188} and Asn^{196} in saporin 5 involve a significant change in polarity or charge of the amino acid residues, while the changes at the other six positions are conservative in nature. We thus proposed that the non-conservative amino acid differences that lie outside the active site of the toxin could affect the local structure, resulting in the differential catalytic activity of the two isoforms [9].

There has been a growing evidence for the action of RIPs on non-ribosomal substrates [11–13]. Saporin 6 has been reported to contain DNA nuclease activity, and saporin L1 has been shown to act on various forms of mammalian DNA [12,14]. While the recombinant forms of ricin and pokeweed antiviral protein purified from *Escherichia coli* had no DNase activity, both native and recombinant gelonin degrade single-stranded DNA [15,16]. Cinnamomin A-chain deadenylates supercoiled double-stranded DNA [17]. We recently showed the overall cytotoxic activity of saporin 6 to be a cumulative effect of its RNA N-glycosidase and DNA fragmentation activities [18].

In the present study, the basis for the differential catalytic activity between the two saporin seed isoforms 5 and 6 was studied by mutating the residues Lys^{134}, Leu^{147}, Phe^{149}, Asn^{162}, Thr^{188} and Asp^{196} in saporin 6 to the corresponding residues Gln^{134}, Ser^{147}, Ser^{149}, Asp^{162}, Ile^{188} and Asn^{196} of saporin 5. Among these non-conservative changes, only the change in amino acid residue from Asn^{162} in saporin 6 to Asp^{162} of saporin 5 significantly reduced the catalytic activity of saporin 6, while the changes at other positions did not affect the catalytic activity of saporin 6. The isoforms 5 and 6 both exerted a Mg^{2+}-dependent DNA nuclease-like activity on plasmid DNA, with saporin 6 being much more potent than saporin 5. The N162D substitution also resulted in a reduction in the DNase activity of saporin 6.

Abbreviations used: RIP, ribosome-inactivating protein; SRL, sarcin–ricin loop.

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Table 1 Amino acid differences between saporin isoforms 5 and 6

The differences between saporin 5 and saporin 6 at various amino acid residues are listed. The right-most column describes the secondary structures in which the respective residues lie, based on the crystal structure of saporin 6 [10]. The numbers in parentheses indicate the residue-numbers that form the particular α-helix. The positions depicted in bold represent those at which there is a non-conservative change in residue between the two isoforms.

	Residue		
Position	Saporin 5	Saporin 6	Structure
48	E	D	Loop
91	R	K	Hydrogen-bonded turn
134	**Q**	**K**	**Hydrogen-bonded turn**
147	**S**	**L**	**α-Helix (142–152)**
149	**S**	**F**	**α-Helix (142–152)**
162	**D**	**N**	**α-Helix (159–173)**
177	A	V	α-Helix (174–180)
188	**I**	**T**	**α-Helix (180–190)**
196	**N**	**D**	**Loop**
198	E	D	α-Helix (198–207)
207	N	S	Hydrogen-bonded turn
209	K	R	α-Helix (207–218)

EXPERIMENTAL

Construction of saporin 6 mutants

Wild-type saporin isoforms 5 and 6, without the signal sequence, consist of 253 amino acid residues. pSap6 and pSap5 are plasmids respectively containing the 759 bp DNA encoding saporin 6 and saporin 5 cloned downstream of a T7 promoter in the bacterial expression vector pVex11 [9]. pSap6 was used as a template to mutate the codons for residues Lys^{134}, Leu^{147}, Phe^{149}, Asn^{162}, Thr^{188} and Asp^{196} of saporin 6 to the codons for glutamine, serine, serine, glutamate, isoleucine and asparagine respectively by oligonucleotide-mediated site-directed mutagenesis [19]. The saporin 6 double mutants in which mutation N162D was combined with each of the other single mutations were constructed by a three-step PCR. The mutations were confirmed by DNA sequencing using the dideoxy chain termination method [20].

Expression and purification of recombinant proteins

The proteins were expressed in BL21 (λDE3) strain of *E. coli*. Bacterial cells were transformed with the desired construct and grown in Superbroth [12 g/l tryptone, 24 g/l yeast extract, 6.3 g/l glycerol, 12.5 g/l K_2HPO_4, 3.8 g/l KH_2PO_4, 0.4 g/l $MgSO_4$ and 4 g/l glucose (pH 7.2)] containing 100 μg/ml ampicillin at 37 °C with shaking. All proteins were found to accumulate in the form of inclusion bodies, and they were purified as described earlier for saporin [9,18]. Briefly, the resuspended cells were lysed with lysozyme, and the membrane pellet was washed with Triton X-100 followed by several washings without Triton X-100. The inclusion body pellet thus obtained was dissolved in guanidine hydrochloride and reduced by adding dithiothreitol. Renaturation was carried out by 100-fold dilution of the protein in a refolding buffer containing 0.1 M Tris/HCl (pH 8.0), 0.5 M L-arginine/HCl, 0.9 mM GSSG and 0.1 mM EDTA. After incubating at 10 °C for 48 h, the renatured material was dialysed against 20 mM sodium acetate buffer (pH 4.5), containing 100 mM urea. The dialysed solution was loaded on an S-Sepharose column and eluted using a 0–2 M gradient of NaCl in 20 mM sodium acetate buffer (pH 4.5). Relevant fractions were pooled, concentrated and purified to homogeneity by gel-filtration chromatography on a Superose-12 column (Amersham Biosciences) in PBS (pH 7.4).

Structural characterization by CD

For CD spectral analysis, 30 μg of protein was dissolved in 600 μl of 10 mM sodium phosphate buffer (pH 7.0), and spectra were recorded in the far-UV range (200–250 nm) at room temperature (25 °C), using a JASCO J710 spectropolarimeter. A cell with a 0.2 cm optical path was used to record the spectra at a scan speed of 200 nm/min with a sensitivity of 50 millidegrees and a response time of 1 s. The sample compartment was purged with nitrogen, and spectra were averaged over ten scans. The results are presented as mean residue molar ellipticity.

Assay for inhibition of *in vitro* protein synthesis

The ability of the saporin isoforms and variants to inhibit protein synthesis was measured using a rabbit reticulocyte lysate-based *in vitro* translation assay system as described in [21]. The reaction mix of a final volume of 30 μl contained 10 μl of rabbit reticulocyte lysate, 1 mM ATP, 0.2 mM GTP, 75 mM KCl, amino acid mix without leucine, 0.16 μCi of [^{3}H]leucine, 1.33 mg/ml creatine kinase, 2.66 mg/ml phosphocreatine and different concentrations of the toxin, diluted in 0.2 % RNase-free BSA. The endogenous globin mRNA in the reticulocyte lysate was used as the template for translation. The reaction was carried out at 30 °C for 1 h and stopped by adding 0.25 ml of 1 M NaOH containing 0.2 % H_2O_2, followed by incubation at 37 °C for 10 min. The proteins were precipitated with 15 % (w/v) trichloroacetic acid on ice for 30 min and harvested on 26 mm glass fibre filters. The dried filters were counted using a liquid-scintillation counter. Activity was expressed as the percentage of control where no toxin was added. ID_{50} represents the concentration of toxin that inhibited *in vitro* protein synthesis by 50 %.

Cytotoxic activity of saporin isoforms and variants

The cytotoxic activity of saporin isoforms and variants was assayed on J774A.1, a mouse monocyte-macrophage adherent cell line. The cells were plated on to a 96-well plate at a density of 5×10^3 cells/well in 0.2 ml of leucine-free RPMI 1640 medium containing 5 % (v/v) foetal calf serum and grown for 16 h. The cells were then incubated with various concentrations of the toxins, diluted in 0.2 % human serum albumin in Dulbecco's PBS for 46 h followed by labelling with 0.75 μCi of [^{3}H]leucine/well for 2 h. After freezing and thawing, the cells were harvested on filtermats using a 96-well plate automated harvester, and the filters were counted in an LKB β-plate counter. Activity was plotted as percentage of control where no toxin was added to the cells, and the results were expressed as ID_{50} values. The ID_{50} values represent the concentration of the toxin that inhibited the cellular protein synthesis by 50 %.

Assay for DNase-like activity of saporin isoforms and variants

The DNase-like activity of saporin isoforms and its variants was studied as described in [17]. Different amounts of the proteins were incubated with 0.5 μg of supercoiled plasmid pBR322 DNA or 2 μg of rat liver genomic DNA, at 37 °C for 1 h in a reaction mix containing 50 mM Tris/HCl (pH 7.5), 50 mM KCl and 10 mM $MgCl_2$, in a final volume of 20 μl. A similar reaction was performed in the absence of $MgCl_2$ or presence of 2 mM EDTA to check the dependence of nuclease-like activity on Mg^{2+} ions [15]. Following incubation, the reaction was stopped by adding the gel loading dye, 2.5 % Ficoll, 0.05 % Bromophenol Blue and 0.05 % Xylene Cyanol. Samples were analysed on a 1 % agarose gel and visualized by ethidium bromide staining. The linear form of pBR322 DNA was prepared by digesting the supercoiled form with HindIII at 37 °C for 3 h.

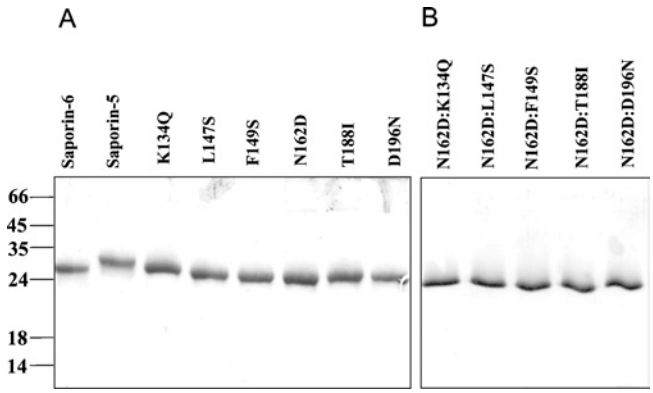

Figure 1 SDS/PAGE of single and double amino acid variants of saporin 6

Both single variant (**A**) and double variant (**B**) proteins were expressed in the BL21 (λDE3) strain of *E. coli* and purified from the inclusion bodies by cation-exchange and gel-filtration chromatography. The recombinant proteins were analysed by SDS/12.5 % PAGE under reducing conditions, followed by Coomassie Blue staining. Molecular-mass markers are indicated in kDa on the left.

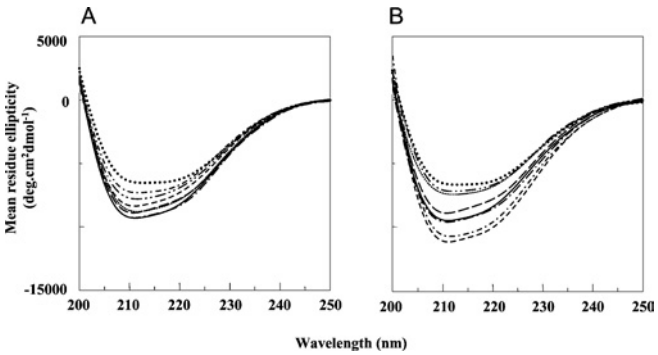

Figure 2 CD-spectral analysis of saporin 5, saporin 6 and single and double amino acid variants of saporin 6

CD spectra were recorded in the far-UV region (200–250 nm) at 25 °C. The spectra are presented as mean residue molar ellipticity, expressed in degree · cm^2/dmol. (**A**) Saporin 6 (——), saporin 5 (●●●●●), K134Q (·····), L147S (- - -), F149S (- · - · -) N162D (— — —), T188I (— · — · —) and D196N (—·· —··). (**B**) Saporin 6 (——), saporin 5 (●●●●●), N162D (— — —), N162D/K134Q (- - -), N162D/L147S (- · - · -), N162D/F149S (·····), N162D/T188I (—·—·—) and N162D/D196N (—·· —··).

RESULTS

Expression and purification of saporin isoforms and single and double amino acid variants

Saporin isoforms 6 and 5 and the various single and double amino acid variants were expressed in BL21 (λDE3) strain of *E. coli* cells, and the overexpressed proteins were found to localize in the inclusion bodies. The recombinant proteins from the inclusion bodies were denatured and refolded *in vitro* and purified by a two-step purification procedure comprising cation-exchange and gel-filtration chromatography. By SDS/PAGE analysis, the single amino acid variants gave a single band at the same position as saporin 6, indicating that the preparations were homogeneous (Figure 1A). Similarly, the double variants N162D/K134Q, N162D/L147S, N162D/F149S, N162D/T188I and N162D/D196N were also found to be homogeneous on SDS/PAGE (Figure 1B). On Western blots, all the variants reacted equally well with a polyclonal antibody raised against saporin 6 (results not shown).

Characterization of proteins by CD spectroscopy

The effect of the various single and double mutations on the overall structure of saporin 6 was studied by CD-spectral analysis of the purified variants in the far-UV region. The native saporin is an $\alpha + \beta$ protein, therefore the CD profile of the protein corresponds to the broad minima over 208–222 nm range, consistent with the structural composition of the protein. Saporin 5 also showed a similar CD spectrum; however, the amplitude of the profile was different from that of saporin 6 (Figures 2A and 2B). The far-UV CD spectra of T188I, N162D, K134Q and L147S variants of saporin 6 did not show major changes in the mean residue molar ellipticity as compared with saporin 6 over this range of wavelengths, whereas the spectra of F149S and D169N variants resembled the spectrum of saporin 5 more closely (Figure 2A). Among the double variants, the spectra of N162D/T188I, N162D/K134Q and N162D/L147S were similar to that of saporin 6, while the spectra of N162D/F149S and N162D/D169N shifted towards that of saporin 5 (Figure 2B). Differences in the amplitudes of the profiles of N162D/K134Q and N162D/L147S could be attributed to changes in the α-helix and β-sheet content of the variants compared with saporin 6 (Figure 2B).

Table 2 *In vitro* protein synthesis inhibitory activity of saporin isoforms and the variants

Rabbit reticulocyte lysate was treated with various concentrations of saporin isoforms or the variants at 30 °C for 1 h. ID_{50} values, shown as means ± S.E.M. from three independent experiments, refer to the concentration of toxin causing 50 % inhibition of protein synthesis. The numbers in parentheses indicate fold difference in activity compared with wild-type saporin 6.

Protein	ID_{50} (ng/ml)
Saporin 6	0.6 ± 0.04 (1)
Saporin 5	10.3 ± 0.33 (17)
K134Q	0.6 ± 0.10 (1)
L147S	0.4 ± 0.04 (0.7)
F149S	0.8 ± 0.12 (1.3)
N162D	6.4 ± 0.31 (11)
T188I	0.8 ± 0.38 (1.3)
D196N	0.8 ± 0.30 (1.3)
N162D/K134Q	5.7 ± 0.44 (10)
N162D/L147S	4.9 ± 0.21 (8)
N162D/F149S	5.8 ± 0.55 (10)
N162D/T188I	6.8 ± 0.33 (11.3)
N162D/D196N	5.9 ± 0.58 (10)

In vitro protein synthesis inhibitory activity of saporin isoforms and various variants

The ability of the saporin isoforms and single and double amino acid variants to inhibit protein synthesis was tested in a rabbit reticulocyte lysate-based *in vitro* translation assay. The decrease in the incorporation of [^{3}H]leucine in the nascent peptides was taken as a measure of protein synthesis inhibition by the toxin. Saporin 6 caused a dose-dependent inhibition of protein synthesis with an ID_{50} of 0.6 ng/ml (Table 2). Saporin 5 had inhibitory activity that was 17-fold less than that of saporin 6; the single amino acid variant N162D had activity 11-fold less than that of saporin 6, while the single amino acid variants K134Q, L147S, F149S, T188I and D196N showed activity that was not significantly different from that of saporin 6 (Table 2).

The double variants N162D/K134Q, N162D/L147S, N162D/F149S, N162D/T188I and N162D/D196N showed protein synthesis inhibitory activity that closely resembled that of N162D (Table 2).

Table 3 Cytotoxic activity of saporin isoforms and variants

J774A.1 cells were treated with different concentrations of saporin isoforms or the variants for 48 h. The incorporation of [^{3}H]leucine was measured in the toxin-treated cells and compared with the control cells where no toxin was added. ID_{50} values, shown as means ± S.E.M. from three independent experiments, refer to the concentration of toxin required to inhibit cellular protein synthesis by 50%. The numbers in parentheses indicate fold difference in activity compared with the wild-type saporin 6.

Protein	ID_{50} (ng/ml)
Saporin 6	4.4 ± 0.15 (1)
Saporin 5	43.0 ± 2.91 (9.8)
K134Q	3.2 ± 0.30 (0.7)
L147S	6.2 ± 0.75 (1.4)
F149S	6.5 ± 0.67 (1.5)
N162D	34.0 ± 0.67 (7.7)
T188I	4.5 ± 0.40 (1)
D196N	4.8 ± 0.35 (1.1)
N162D/K134Q	33.0 ± 0.33 (7.5)
N162D/L147S	30.0 ± 0.89 (6.8)
N162D/F149S	31.0 ± 0.89 (7.0)
N162D/T188I	32.0 ± 0.33 (7.3)
N162D/D196N	31.0 ± 0.33 (7.0)

Cytotoxic activity of saporin isoforms and variants

The mouse monocyte-macrophage cancer cell line J774A.1 was treated with different concentrations of saporin 6, saporin 5 or the single and double amino acid variants, and protein synthesis inhibition in the cells was taken as the measure of cytotoxicity. Saporin 6 exerted a dose-dependent toxicity on J774A.1 cells, with an ID_{50} of 4.4 ng/ml (Table 3). The variant N162D was approx. 8-fold less toxic than saporin 6, while the other single amino acid variants had toxicities comparable with that of saporin 6 (Table 3). Saporin 5 was approx. 10-fold less active compared with saporin 6 (Table 3). The double variants comprising N162D had cytotoxicities similar to that of the single amino acid variant N162D (Table 3).

DNase-like activity of saporin isoforms and variants

The DNase-like activity of the saporin isoforms and N162D variant was tested on supercoiled plasmid pBR322 DNA as well as on rat liver genomic DNA. In the presence of Mg^{2+} ions, supercoiled plasmid DNA was converted into linear and nicked forms by saporin 6 at lower concentrations of protein, while at higher concentrations, all forms of DNA were completely degraded (Figure 3). Saporin 5 at high concentrations converted the entire supercoiled DNA into linear and nicked forms, but no DNA degradation was observed (Figure 3). The variant N162D showed a profile very similar to that of saporin 5 (Figure 3).

To ascertain the requirement of Mg^{2+} ions for DNA-nuclease-like activity of saporin 6, plasmid DNA was incubated in the absence of Mg^{2+} ions, and also in the presence of 2 mM EDTA that would chelate any Mg^{2+} ions inherently present in the protein preparations. A significant lowering of the DNA nuclease-like activity was observed for saporin 6, saporin 5 and N162D variant in the absence of Mg^{2+} ions (Figure 3). The activity was totally abolished in the absence of Mg^{2+} and presence of 2 mM EDTA (Figure 3).

When genomic DNA was used as the substrate, it was observed that 2 μg of the DNA could be completely degraded into smaller fragments by 5 μg of saporin 6, while no significant degradation was caused by saporin 5 and N162D variant (Figure 4).

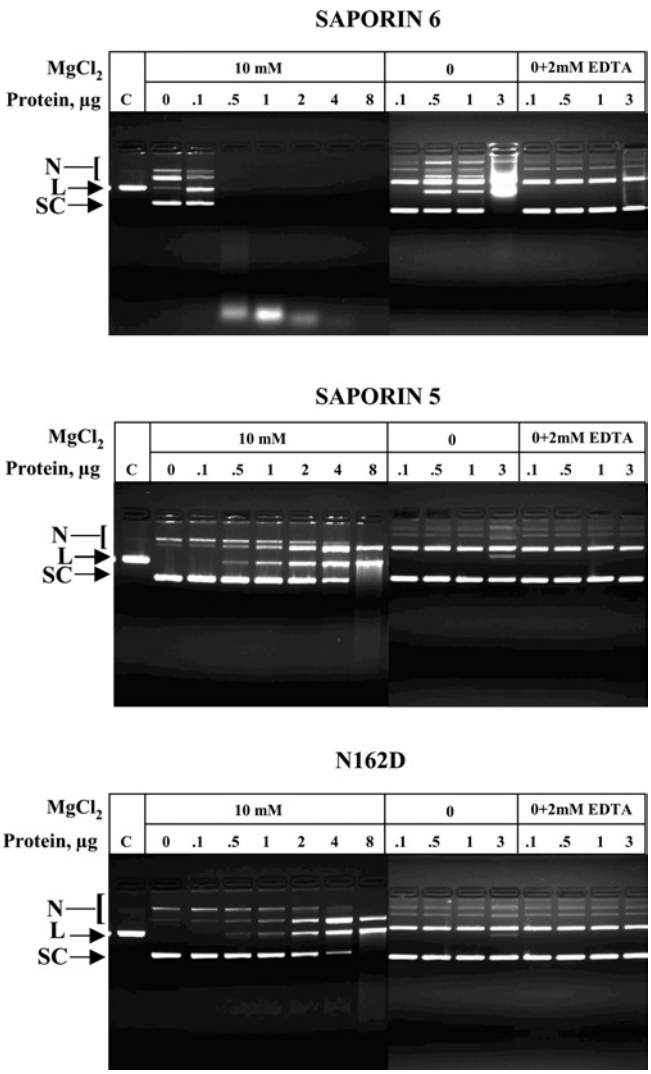

Figure 3 Nuclease activity of saporin isoforms and N162D variant on supercoiled plasmid DNA

Plasmid DNA (0.5 μg) was incubated with indicated amounts of the respective proteins, in the presence or absence of $MgCl_2$ and EDTA. The reaction was stopped by adding loading dye. The samples were resolved on a 1% agarose gel and visualized by ethidium bromide staining. N, L and SC represent nicked, linear and supercoiled DNA respectively.

DISCUSSION

Saporin 6 has been shown to have at least a 10-fold higher N-glycosidase activity than saporin 5 that results in a similar difference in their cytotoxic activities [9]. Since the active site residues in the two isoforms are conserved, the difference in the activity of the two isoforms could be attributed to amino acid differences, which lie outside the active site of the toxin. The present study was conducted to elucidate whether non-conservative changes in amino acid residues between the two saporin seed isoforms 6 and 5 that occur outside the catalytic sites play a role in the differential catalytic activities of these isoforms. The residues Lys^{134}, Leu^{147}, Phe^{149}, Asn^{162}, Thr^{188} and Asp^{196} of saporin isoform 6 all lie outside the active site of the protein and are replaced in saporin isoform 5 by the residues Gln^{134}, Ser^{147}, Ser^{149}, Asp^{162}, Ile^{188} and Asn^{196} respectively. These residues of saporin 6 were mutated to the corresponding residues of saporin 5. Mutating Asn^{162} in saporin 6 to Asp^{162} of saporin 5 caused a significant reduction of both

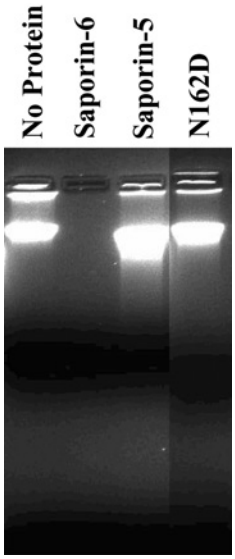

Figure 4 Nuclease activity of saporin isoforms and N162D variant on genomic DNA

Rat liver genomic DNA (2 μg) was incubated with 5 μg each of the respective proteins in the presence of $MgCl_2$. The reaction was stopped by adding loading dye. The samples were resolved on a 1 % agarose gel and visualized by ethidium bromide staining.

in vitro protein synthesis inhibitory activity as well as cytotoxicity of saporin 6. The N162D variant of saporin 6 and saporin 5 had similar activity. The mutations K134Q, L147S, F149S, T188I and D196N had no effect on the catalytic activity or cytotoxicity of saporin 6. The combination of mutation N162D with each of the other point mutations did not decrease the activity of N162D any further. The study indicates that the non-conservative amino acid changes at positions 134, 147, 149, 188 and 196 between saporin isoforms 6 and 5 play no role in causing differential catalytic activity between these isoforms, while the change in Asn^{162} in saporin 6 to an aspartate residue in saporin 5 was responsible for most of the reduction in the activity of this isoform. The DNA nuclease-like activity of saporin isoforms 5 and 6 and the N162D variant on pBR322 and rat genomic DNA also matched with their RNA N-glycosidase activity.

The residues proposed to be present at the active site cleft of saporin, Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179} and W^{208}, are identical in both the isoforms [10]. Saporin 6 differs from saporin 5 at 12 positions (Table 1). Based on the crystal structure of saporin 6, the secondary structures in which these residues lie are given in Table 1 [10]. It should be noted that none of these residues lies in β-sheet and most of the changes in saporin 5 compared with saporin 6 are either in α-helical regions or in β-turns. Of the 12 differences between saporin 5 and saporin 6, changes at six positions, 134, 147, 149, 162, 188 and 196, result in a change in polarity or charge. It is likely that these differences are responsible for the observed difference in the activity of the two isoforms. Based on a putative model of saporin, Fabbrini et al. [22] had postulated the substitution of Lys^{134} of seed-type saporin with a glutamine residue in the leaf cDNA isoform to be responsible for their difference in activity. This residue was predicted to be located at a conserved surface loop of RNA-binding domain. The crystal structure of saporin 6 showed that Lys^{134} is present in a hydrogen-bonded turn and Asp^{196} is present in a loop. Substitution of the charged amino acids Lys^{134} or Asp^{196} in saporin 6 with the uncharged amino acids glutamine or asparagine of saporin 5 respectively may result in a change in local structure as well as charge, which can affect the toxin interactions and activity on the ribosomes. However, in the present study, we did not find the K134Q mutation to be detrimental for the activity of saporin 6. On the other hand, substitution of an uncharged asparagine residue with aspartic acid at position 162 resulted in a significant reduction in the activity of saporin 6. Asn^{162} lies in the α-helix, spanning residues 159–171, which is parallel and opposite to the α-helix spanning residues 142–152. Asn^{162} is in close proximity to Phe^{149}, Ala^{151} and Val^{153} of the opposite α-helix. The substitution of asparagine to aspartic acid could weaken the general stability as it will bring extra negative charge in the close vicinity of the three hydrophobic residues from the other helix and may therefore affect the stability of the active site.

Although, the mechanism underlying the catalytic activity of RIPs is understood, very little is known about how RIPs gain access to the ribosome. The ricin A chain was found to cross-link to mammalian ribosomal proteins L9 and L10e [23], whereas pokeweed antiviral protein gains access to the ribosome by recognizing L3 [24]. Ippoliti et al. [25] have shown, by chemical cross-linking of saporin to yeast ribosomal proteins, that at least one ribosomal protein of 30 kDa from the 60 S subunit comes into contact with saporin and is cross-linked. In the crystal structure of the 50 S ribosomal subunit, 23 S rRNA and five proteins, L3, L6, L13, L14 and L24e, comprise the surface near the SRL [26,27]. These proteins have bipolar character, with most of the basic residues buried inside the ribosome and acidic residues exposed to solvent [28]. Many of these acidic residues are conserved among bacterial, archaeal and eukaryotic ribosomes. Recently, it has been shown that the electrostatic character of ribosomal surface enables restrictocin, a fungal ribonuclease that targets the same tetranucleotide in SRL as RIPs, to bind at many sites on the ribosomal surface and diffuse within the ribosomal electrostatic field to the SRL [29]. Savino et al. [10], based on studies on molecular interaction between saporin and the yeast ribosome by differential chemical modifications, have identified a contact surface inside the C-terminal region of saporin. Structural comparison between saporin and other ribosome-inactivating proteins revealed this region to be conserved and represent a peculiar motif involved in ribosome recognition [10]. Although Asn^{162} does not fall in the C-terminal region, its substitution with an aspartic acid residue in saporin 6 would result in extra negative charge in the molecule, which may have implications in the interaction of the protein with ribosomal protein(s), a prerequisite for binding to ribosomes resulting in reduced translational inhibitory and cytotoxic activities.

In conclusion, we show that the reduced activity of saporin 5 compared with saporin 6 is due to a single amino acid substitution of Asn^{162} in saporin 6 with Asp^{162} in saporin 5. This substitution results in a local structural change in the molecule resulting in a reduction in its catalytic activity both on RNA and DNA substrates and also in the cytotoxicity of the protein.

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REFERENCES

1 Barbieri, L., Batelli, M. G. and Stirpe, F. (1993) Ribosome-inactivating proteins from plants. Biochim. Biophys. Acta **1154**, 237–282

2 Endo, Y., Tsurugi, K., Yutsudo, T., Takeda, Y., Ogasawara, T. and Igarashi, K. (1988) Site of action of a Vero toxin (VT2) from *Escherichia coli* O157:H7 and of shiga toxin on eukaryotic ribosomes: RNA N-glycosidase activity of the toxins. Eur. J. Biochem. **171**, 45–50

3 Endo, Y., Gluck, A. and Wool, I. G. (1991) Ribosomal RNA identity elements for ricin A-chain recognition and catalysis. J. Mol. Biol. **221**, 193–207
4 Irvin, J. D. (1983) Pokeweed antiviral protein. Pharmacol. Ther. **21**, 371–387
5 Stirpe, F. and Barbieri, L. (1986) Ribosome-inactivating proteins up to date. FEBS Lett. **195**, 1–8
6 Stirpe, F., Gasperi-Campani, A., Barbieri, L., Falasca, A., Abbondaza, A. and Stevens, W. A. (1983) Ribosome-inactivating proteins from seeds of *Saponaria officinalis* L. (soapwort), of *Agrostemma githago* L. (corn cockle) and of *Asparagus officinalis* L. (asparagus) and from the latex of *Hura crepitians* L. (sandbox tree). Biochem. J. **216**, 617–625
7 Benatti, L., Saccardo, M. B., Dani, M., Nitti, G., Sassano, M., Lorenzatti, R., Lappi, D. A. and Soria, M. (1989) Nucleotide sequence of cDNA coding for saporin-6, a type I ribosome-inactivating protein from *Saponaria officinalis*. Eur. J. Biochem. **183**, 465–470
8 Ferreras, J. M., Barbieri, L., Girbed, T., Batelli, M. G., Rojo, A. M., Arias, F. J., Rocher, M. A., Soriano, F., Mendez, E. and Stirpe, F. (1993) Distribution and properties of major ribosome-inactivating proteins of plant *Saponaria officinalis*. Biochem. Biophys. Acta **1216**, 31–42
9 Bagga, S., Hosur, M. V. and Batra, J. K. (2003) Cytotoxicity of ribosome-inactivating protein saporin is not mediated through α_2-macroglobulin receptor. FEBS Lett. **541**, 16–20
10 Savino, C., Federici, L., Ippoliti, R., Lendaro, E. and Tsernoglou, D. (2000) The crystal structure of saporin SO6 from *Saponaria officinalis* and its interaction with ribosomes. FEBS Lett. **470**, 239–243
11 Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Polynucleotide:adenosine glycosidase activity of ribosome-inactivating proteins: effect on DNA, RNA and poly(A). Nucleic Acids Res. **25**, 518–522
12 Barbieri, L., Valbonesi, P., Govoni, M., Pession, A. and Stirpe, F. (2000) Polynucleotide:adenosine glycosidase activity of saporin L1: effects on various forms of mammalian DNA. Biochim. Biophys. Acta **1480**, 258–266
13 Hudak, K. A., Wang, P. and Tumer, N. E. (2000) An insight into the mechanism of cytotoxicity of ricin to hepatoma cell: roles of bcl-2 family proteins, caspases, Ca^{2+} dependent proteases and protein kinase C. RNA **6**, 369–380
14 Roncuzzi, L. and Gasperi-Campani, A. (1996) DNA-nuclease activity of single chain ribosome-inactivating proteins dianthin-30, saporin-6 and gelonin. FEBS Lett. **392**, 16–20
15 Day, P. J., Lord, M. and Roberts, L. M. (1998) The deoxyribonuclease activity attributed to ribosome-inactivating proteins is due to contamination. Eur. J. Biochem. **258**, 540–545
16 Nicolas, E., Beggs, J. M., Haltiwanger, B. M. and Taraschi, T. F. (1997) Direct evidence for the deoxyribonuclease activity of the plant ribosome inactivating protein gelonin. FEBS Lett. **406**, 162–164
17 He, W. and Liu, W. (2004) Both N- and C-terminal regions are essential for cinnamomin A chain to deadenylate ribosomal RNA and supercoiled double-stranded DNA. Biochem. J. **377**, 17–23
18 Bagga, S., Seth, D. and Batra, J. K. (2003) The cytotoxic activity of ribosome-inactivating protein saporin is attributed to its rRNA N-glycosidase and internucleosomal DNA fragmentation activities. J. Biol. Chem. **278**, 4813–4820
19 Kunkel, T. A., Roberts, J. D. and Zakour, R. A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154**, 367–382
20 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**, 5463–5467
21 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning. A Laboratory Manual, 2nd edn, vol. 3, pp. 18.76–18.80, Cold Spring Harbor Laboratory Press, Cold Spring Harbor
22 Fabbrini, M. S., Rappocciolo, E., Carpani, D., Solinas, M., Valsasino, B., Brime, U., Cavallaro, U., Nykjaer, A., Rovida, E., Legname, G. and Soria, M. R. (1997) Characterization of a saporin isoform with lower ribosome-inactivating activity. Biochem. J. **322**, 719–727
23 Vater, C. A., Bartle, L. M., Leszyk, J. D., Lambert, J. M. and Goldmacher, V. S. (1995) Ricin A chain can be chemically cross-linked to the mammalian ribosomal proteins L9 and L10e. J. Biol. Chem. **270**, 12933–12940
24 Hudak, K. A., Dinman, J. D. and Tumer, N. E. (1999) Pokeweed antiviral protein accesses ribosomes by binding to L3. J. Biol. Chem. **274**, 3859–3864
25 Ippoliti, R., Lendaro, E., Bellelli, A. and Brunori, M. (1992) A ribosomal protein is specifically recognized by saporin, a plant toxin which inhibits protein synthesis. FEBS Lett. **298**, 145–148
26 Ban, N., Nissen, P., Hansen, J., Moore, P. B. and Steitz, T. A. (2000) The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. Science **289**, 905–920
27 Klein, D. J., Schmeing, T. M., Moore, P. B. and Steitz, T. A. (2001) The kink-turn: a new RNA secondary structure motif. EMBO J. **20**, 4214–4221
28 Moore, P. B. and Steitz, T. A. (2003) The structural basis of large ribosomal subunit function. Annu. Rev. Biochem. **72**, 813–850
29 Korennykh, A. V., Piccirilli, J. A. and Correll, C. C. (2006) The electrostatic character of the ribosomal surface enables extraordinarily rapid target location by ribotoxins. Nat. Struct. Mol. Biol. **13**, 436–443

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