The cytotoxic activity of ribosome-inactivating protein saporin-6 is attributed to rRNA N-glycosidase and internucleosomal DNA fragmentation

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**SUMMARY**

Saporin-6 produced by the plant *Saponaria officinalis* belongs to the family of single chain ribosome-inactivating proteins. It potently inhibits protein synthesis in eukaryotic cells, by cleaving the N-glycosidic bond of a specific adenine in 28S rRNA, which results in the cell death. Saporin-6 has also been shown to be active on DNA, and induces apoptosis. In the current study we have investigated the roles of rRNA depurination, and the activity of saporin-6 on genomic DNA in its cytotoxic activity. The role of putative active site residues Y72, Y120, E176, R179 and W208, and two invariant residues Y16 and R24, proposed to be important for structural stability of saporin-6 has been investigated in its catalytic and cytotoxic activity. These residues were mutated to alanine to generate seven mutants Y16A, R24A, Y72A, Y120A, E176A, R179A and W208A. We show that for the RNA N-glycosidase activity of saporin-6, residues Y16, Y72 and R179 are absolutely critical; Y120 and E176 can be partially dispensed with, whereas W208 and R24 do not appear to be involved in this activity. The residues Y72, Y120, E176, R179 and W208 were found to be essential for the genomic DNA fragmentation activity, whereas residues Y16 and R24 do not appear to be required for the DNA fragmentation. The study shows that saporin-6 possesses two catalytic activities, namely RNA N-glycosidase and genomic DNA fragmentation activity, and for its complete cytotoxic activity both activities are required.
INTRODUCTION

Ribosome-inactivating proteins (RIPs) are toxic translation inhibitors produced by plants and bacteria. RIPs have been classified into two types, Type I RIPs are composed of a single polypeptide chain, while type II RIPs consist of two chains, A and B, linked by a disulphide bond (1). Type I RIPs e.g. saporin, trichosanthin and pokeweed antiviral protein (PAP), and A-chain of type II RIPs e.g. ricin and abrin inhibit protein synthesis by removing a specific adenine from 28SrRNA of eukaryotic ribosomes. The former also removes an equivalent adenine residue, A2660, from 23SrRNA of *E. coli* ribosomes (2). In both the cases, the site of action is located in a highly conserved, purine rich stem and loop structure of rRNA termed as the α-sarcin/ricin loop (3). The catalytic depurination disrupts the binding of elongation factors to the ribosomes, thus arresting protein synthesis at the translocation step (4). Although the catalytic action carried out by all the RIPs is identical, their activity on ribosomes from sources other than eukaryotes are markedly different (1,5). Differences in toxicity of RIPs towards various cell lines, their different requirements for cofactors and variations in the minimal structure of the adenine containing loop which they can attack point to their substantial diversity (6,7). The study of molecules, which bind and inactivate RIPs has also suggested that local sequence/structure variabilities exist among RIPs (8). The interest in RIPs has gained a new momentum recently with the growing evidence of their action on non-ribosomal substrates (9-11). Most of the novel enzymatic activities are related to a presumed RNase or DNase activity. Other enzymatic activities reported for individual RIPs include phosphatase activity on lipids, chitinase activity and superoxide dismutase activity (12-14). It has been shown that PAP cleaves the double stranded supercoiled DNA using the same active site required to depurinate rRNA, and that momordin (MOM) has intrinsic RNase activity (15,16). A better understanding of the catalytic mechanism of RIPs
will be extremely useful in the exploitation of their unique properties for diverse applications like development of RIP-based immunotoxins, abortifacients and anti-HIV agents (17).

Saporin is a family of single-chain ribosome-inactivating proteins present in abundance in the plant *Saponaria officinalis* (18). Among RIPs, various peculiar features of saporin, in terms of its remarkable stability and activity on a wide variety of substrates make it an interesting protein to study for structure-function relationships (19,20). The crystal structure of saporin-6 has been solved recently (21). The structure superimposition of saporin-6 has shown that corresponding to ricin A-chain active site residues, the residues Tyr72, Tyr120, Glu176, Arg179 and Trp208 constitute the active site of saporin-6 (21,22).

In the present study analytical mutagenesis of saporin-6, the most active isoform, was carried out to study the role of residues present in its active site. The active site residues Y72, Y120, E176, R179 and W208 were mutated to alanine and the activity of the mutants were compared to the wild type protein using various functional assays. An interesting feature found in primary sequence alignment of RIPs was the invariance of residues, tyrosine and arginine, corresponding to position 21 and 29 respectively in ricin sequence. An analysis of ricin-A chain structure showed that a central $\alpha$-helix is bent near its C-terminus and this bending allows the catalytic residues, Glu177 and Arg180, to reach the solvent of the active site cleft (23). This important helix bending disrupts the normal $\alpha$-helical bonding pattern. However, the resulting structure is stabilized by new hydrogen bonds to the side chains of Tyr21 and Arg29. Alterations of these residues could, therefore, affect the folding rate or thermodynamic stability of the protein. In order to ascertain the role of corresponding residues, Tyr16 and Arg24, in saporin-6 they were mutated to alanine and the mutants were analyzed for various functional activities. Saporin-6 has been reported to contain DNA nuclease activity and recently saporin-L1 has been shown to act on various forms of mammalian DNA (10,24). DNA could be a probable alternate polynucleotide substrate for
RIPs within a cell. In the present study the effect of saporin-6 and the mutants has been studied on genomic DNA of U937 cells. An attempt has been made to correlate the cytotoxicity of saporin-6 with the effect of toxin action on DNA and RNA. Our study shows that the cytotoxic activity of saporin is a cumulative effect of its RNA N-glycosidase and DNA fragmentation activity.
EXPERIMENTAL PROCEDURES

Construction of Saporin-6 mutants

Saporin-6 is a protein consisting of 253 amino acid residues. pSap-6 is a plasmid containing the 759 base-pair saporin-6 gene cloned downstream of a T7 promoter in bacterial expression vector pVex 11. pSap-6 was used as a template to mutate the codons for active site residues Tyr72, Tyr120, Glu176, Arg179 and Trp208 to that for alanine. Similarly, the codons for the invariant residues Tyr16 and Arg24 were also mutated to that for alanine. All the mutations were carried out by oligonucleotide-mediated site-directed mutagenesis (25). Uracil containing DNA template was prepared by infecting CJ236 strain of \textit{E. coli} cells with the recombinant phage and growing it in the presence of uridine and chloramphenicol (25). Mutagenesis was performed using the DNA primers JKB54, JKB55, JKB56, JKB57, JKB58, JKB59 and JKB60 containing the mutations Y16A, R24A, Y72A, Y120A, E176A, R179A and W208A respectively. The sequences of various primers used are mentioned in Table 1. The primer extension products were transformed into \textit{E. coli} strain DH5α by standard methods. All mutations were confirmed by DNA sequencing using the dideoxy chain termination method (26).

Expression and purification of the recombinant proteins

Saporin-6 and the mutants were expressed in BL21 (λDE3) strain of \textit{E. coli}. Bacterial cells were transformed with the desired construct and grown in Super broth containing 100 µg/ml ampicillin, at 37°C with shaking. Saporin-6 and all the mutants were found to accumulate in the form of inclusion bodies, and they were purified using the procedure described by Buchner \textit{et al} (27). 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 dithioerythritol. Renaturation was carried out
by 100-folds dilution of the protein in a refolding buffer containing L-arginine and oxidized glutathione. After incubating at 10°C for 48 hours, the renatured material was dialyzed against 20 mM acetate buffer, pH 4.5 containing 100 mM urea. The dialyzed solution was loaded on an S-sepharose column, and eluted using a 0-1.5 M gradient of NaCl in 20 mM acetate buffer, pH 4.5. Relevant fractions were pooled, concentrated and purified to homogeneity by gel filtration chromatography on a TSK 3000 column in PBS, pH 7.4.

**Structural characterization by circular dichroism**

For CD-spectral analysis, 200 µg of protein was dissolved in 3 ml of 10 mM sodium phosphate buffer (pH 7.0) and spectra were recorded in the far-UV range (200-250 nm) at room temperature, using a JASCO J710 spectropolarimeter. A cell with a 1 cm optical path was used to record the spectra at a scan speed of 50 nm/minute with a sensitivity of 50 mdegree and a response time of 1 second. The sample compartment was purged with nitrogen, and spectra were averaged over 10 scans. The results are presented as mean residue ellipticity. Yang’s reference parameters were used to perform secondary structure analyses from CD measurements (28).

**Specific RNA N-glycosidase activity of saporin-6 and the mutants**

The RNA N-glycosidase activity of saporin and its mutants was evaluated as their ability to specifically depurinate 28S rRNA and produce a characteristic 390-base fragment on aniline treatment. Rabbit reticulocyte lysate was taken as the source of ribosomes and treated with different concentrations of proteins at 30°C for half an hour as described by May et al (29). The reaction was stopped by adding 10 µl of 10 % SDS solution and 170 µl of water and incubated at room temperature for 5 minutes. Total RNA was isolated using Trizol reagent as per manufacturer instructions. The RNA pellet was dissolved in 20 µl of water and divided in two parts. To one part, 10 µl of freshly prepared 2 M aniline-acetate, pH 4.5 was added. The samples were incubated at 60°C for 3 minutes, aniline was evaporated under
vacuum and the treated RNA was dissolved in 10 µl of water. To the aniline treated and untreated samples buffer containing 32% formamide, 4 mM EDTA, 0.04% xylene cyanol and 0.04% bromophenol blue were added. The samples were heated at 65°C for 5 minutes and analyzed on a 2% agarose gel. The RNA was visualized by ethidium bromide staining.

**Assay for *in vitro* protein synthesis inhibition**

The capacity of saporin and its mutants to inhibit protein synthesis was measured using a rabbit reticulocyte lysate based *in vitro* translation assay system. The rabbit reticulocyte lysate was prepared and assay carried out as described (30). The reaction mix in a final volume of 30 µl contained 10 µl of rabbit reticulocyte lysate, 1 mM ATP, 0.2 mM GTP, 75 mM KCl, 2 mM magnesium acetate, 3 mM glucose, 10 mM Tris-HCl pH 7.6, 4 µM amino acid mix without leucine, 0.16 µCi [³H] leucine, 1.33 mg/ml creatine phosphokinase, 2.66 mg/ml creatine phosphate and different concentrations of the toxin, diluted in 0.2% RNase free BSA. The endogenous globin mRNA in the reticulocyte lysate was used for translation. The reaction was carried out at 30°C for an hour, stopped by adding 0.25 ml of 1 N NaOH containing 0.2% H₂O₂, followed by an incubation at 37°C for 10 minutes. The proteins were precipitated with 15 % trichloroacetic acid on ice for 30 minutes and harvested on 26 mm glass fiber filters. The dried filters were counted using a liquid scintillation counter. Activity was expressed as percentage of control where no toxin was added. ID₅₀ represents the concentration of toxin that inhibited *in vitro* protein synthesis by 50%.

**Cytotoxic activity of saporin-6 and the mutants**

The cytotoxic activity of saporin-6 and its mutants was assayed on four different cell lines – U937 (human histiocyte lymphoma), L929 (mouse fibroblast), J774A.1 (mouse monocyte-macrophage) and HUT102 (human cutaneous T-cell lymphoma). Adherent cells were plated at a density of 5 x 10³ cells/well in a 96-well plate in 0.2 ml of RPMI/DMEM containing 10% FCS for 16 hours. The medium was replaced with 0.2 ml leucine-free medium.
containing 2% FCS for evaluating the cytotoxicity. The suspension cells were seeded at $10^4$
cells/well in 0.2 ml of leucine-free medium containing 2% FCS and used immediately. The
cells were incubated with various concentrations of toxins, diluted in 0.2% human serum
albumin (HSA) in DPBS for 34 hours followed by labeling with 0.75 µCi $[^3]$H-leucine per well
for 2 hours. The cells after freezing and thawing were harvested on filtermats using a 96-well
plate automated harvester, and the filters were counted using a LKB β-plate counter. Activity
was plotted as percentage of control where no toxin was added to the cells and the results were
expressed in the form of ID$_{50}$ values. The ID$_{50}$ values represent the concentration of the toxin
that inhibited the cellular protein synthesis by 50%.

**Assay for genomic DNA fragmentation**

U937 cells were used to evaluate genomic DNA fragmentation ability of saporin-6
and the mutants. $5 \times 10^5$ cells were cultured in RPMI containing 10% FCS in the presence of
different concentrations of proteins. After indicated time of incubation, cells were harvested
and lysed with 0.2 ml of lysis-buffer consisting of 10 mM Tris-Cl pH 8.0, 100 mM NaCl, 25
mM EDTA pH 8.0, 0.5% SDS and 0.1 mg/ml proteinase K at 50°C for 16 hours. The DNA
was extracted with phenol:chloroform and precipitated with isopropanol. After treating with
RNase A, the DNA samples were run in a 1.5% agarose gel and visualized by staining with
ethidium bromide.

**Intracellular localization of saporin**

Saporin-6 was iodinated using iodogen method as described by Harlow and Lane
(31). 10 µg of protein (0.2-1.0 mg/ml in PBS), 25 µl of iodination buffer and 1 mCi Na$^{125}$I
were added to the iodogen-coated tube. The tube was tapped at room temperature for 10
minutes followed by addition of 50 µl of 0.2% KI. The labeled protein was purified using
PD-10 column (Pharmacia) pre-saturated with BSA.
J774A.1 cells, 6 x 10^6 were seeded in 6 ml of DMEM containing 10% FCS in a T-25 tissue culture flask and incubated at 37°C for 16 hours. The medium was replaced with 1 ml DMEM containing 2% FCS, and cells were incubated for various time intervals with 100 µl (~10^7 cpm) of iodinated saporin-6. After incubation, the cells were washed once with PBS and further incubated for an hour so that membrane bound saporin-6 would either dissociate or internalize. Cells were fractionated into nuclear, cytosolic and membrane fractions as described by Dignam et al. (32). The cell pellet was resuspended in 500 µl buffer containing 10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin, and incubated on ice for 10 minutes. The cells were centrifuged at 3000 x g, resuspended in 0.5 ml of the same buffer and homogenized using a motor-driven homogenizer (30 strokes at 1500 rpm). The homogenate was checked microscopically for cell lysis and centrifuged for 10 minutes at 3000 x g at 4°C. The pellet constituted the nuclear fraction and the supernatant contained cytosol and membranes. To the supernatant 0.11 volume of buffer containing 0.3 M HEPES pH 7.9, 1.4 M KCl and 0.03 M MgCl₂ was added and the mixture was centrifuged at 100,000 x g for 30 minutes at 4°C in an ultracentrifuge. The supernatant and the pellet constituted cytosolic and membrane fractions respectively. The nuclear and membrane pellets were resuspended in 500 µl PBS. The proteins in various fractions were precipitated with 1 ml of 20% TCA. The precipitated proteins were boiled in SDS loading dye for 3 minutes and analyzed on a 12.5% SDS-polyacrylamide gel. The gel was dried, exposed to an X-ray film and later developed.
RESULTS AND DISCUSSION

The present study evaluates the role of various invariant residues present in the ribosome-inactivating protein saporin-6 in its catalytic and cytotoxic activity. These include Y72, Y120, E176, R179 and W208, which have been proposed to be present in the active site and play an important role in the catalysis (21). The role of two other invariant residues Y16 and R24, proposed to be important for structural stability of ricin (33), has also been studied. These residues were mutated to alanine to generate seven single mutants Y16A, R24A, Y72A, Y120A, E176A, R179A and W208A. The target residues were substituted with alanine since it does not impose new hydrogen bonding, sterically bulky, or unusually hydrophobic side chains (34).

Expression, purification and structural characterization of saporin-6 mutants

The mutants of saporin-6 were expressed in BL21 (λDE3) strain of E. coli cells, and the over-expressed mutant proteins were found to localize in the inclusion bodies like the wild type protein. The recombinant proteins from the inclusion bodies were denatured and refolded in vitro, and purified by a two step purification scheme comprising of a cation-exchange and gel filtration chromatography. By SDS-PAGE analysis the mutants gave a single band at the same position as saporin-6 indicating the preparations to be homogeneous (Figure 1). On Western blots, all the mutants reacted equally well with a polyclonal antibody raised against saporin-6 (Figure 1).

The effect of the mutation on the overall structure of saporin-6 was studied by CD-spectral analysis of purified mutants in the far-UV region. Saporin-6 showed the spectra characteristic of \( \alpha + \beta \) structure (Figure 2). The substitution of active site residues Y72, E176 and W208 with alanine resulted in a modest shift of the CD-spectrum whereas the mutants Y120A and R179A appeared to have similar spectra as that of saporin-6 (Figure 2A and 2B). The replacement of the invariant residue Y16 with alanine resulted in a significant shift in the
CD-spectra (Figure 2C). The $\alpha$-helical content of the mutants Y16A, Y72A, Y120A, E176A, R179A and W208A was found to be similar to that of saporin-6, however, there was a decrease in $\beta$-sheet content of these mutants compared to the native toxin (Table 2). The mutant R24A appeared to be similarly folded as the native toxin (Figure 2C). The quantitative values of various secondary structures of R24A were found to be similar to saporin-6 (Table 2).

**RNA N-glycosidase activity of saporin-6 mutants**

The effect of substitution of various active site and invariant residues with alanine was evaluated on the specific RNA N-glycosidase activity of saporin-6. Rabbit reticulocyte lysate was treated with various concentrations of the mutants, and total RNA was extracted. Half of the extracted RNA was treated with aniline, and both aniline-treated and untreated RNA samples were run on an agarose gel. As shown in Figure 3, aniline treatment of saporin-6 treated RNA samples resulted in the release of the classic 390-base Endo-fragment. While saporin-6 produced the Endo-fragment at as low as 40 ng/ml, the mutants Y72A and Y16A failed to affect rRNA even up to 1000 ng/ml (Figure 3). With the mutants Y120A, E176A and R179A a faint Endo-fragment was seen at 1000 ng/ml in the aniline treated samples (Figure 3). Substitution of W208 or R24 with alanine did not have any effect on the RNA N-glycosidase activity of saporin-6 as the mutants W208A and R24A released the Endo-fragment from 28S rRNA at concentrations similar to that of the native toxin (Figure 3). The intensity of Endo-fragment increased with increasing concentrations of the toxin indicating a dose-dependent response. The decrease in size of 28S rRNA upon release of the 390-base fragment was also apparent in aniline treated samples of the mutants W208A and R24A (Figure 3).

The release of Endo-fragment from 28SrRNA as a result of saporin action results in potent inhibition of protein synthesis. The ability of the saporin-6 mutants to inhibit protein
synthesis was tested in a rabbit reticulocyte lysate-based *in vitro* translation assay. The decrease in the incorporation of \[^3\text{H}\]-leucine in the nascent peptides was taken as the measure of protein synthesis inhibition by the toxin. Saporin-6 caused a dose-dependent inhibition of protein synthesis with an ID\textsubscript{50} of 4.5 ng/ml (Table 3). The mutations of W208 and R24 did not affect the protein synthesis inhibitory activity of saporin-6 and mutants W208A and R24A also showed a dose dependent inhibition of protein synthesis with respective ID\textsubscript{50}s of 6.0 ng/ml and 5.8 ng/ml (Table 3). The substitution of invariant residue Y16 and the active site residue Y72 with alanine abolished the protein synthesis inhibitory activity of the protein and even in the presence of 1000 ng/ml of Y16A or Y72A, there was no inhibition of protein synthesis (Table 3). The mutants Y120A and E176A, although caused protein synthesis inhibition but at a relatively higher concentrations compared to the native toxin. The respective ID\textsubscript{50}s of Y120A and E176A were 480 ng/ml and 100 ng/ml, showing these mutants to be 100- and 20-fold less active than the native toxin (Table 3). The mutant R179A showed an extremely poor protein synthesis inhibitory activity, its ID\textsubscript{50} was found to be 200-fold less than the native toxin (Table 3). The reduced protein synthesis inhibitory activity of the mutants is in agreement with the inability or reduced activity of these mutants to generate the Endo-fragment.

Thus, it appears that among the active site residues, Y72 and R179 are absolutely essential for the RNA N-glycosidase activity of saporin-6. The residues Y120 and E176 can be partially dispensed with, whereas W208 and R24 are not required for the RNA N-glycosidase activity. The invariant residue Y16 also appears to be critical for the RNA N-glycosidase activity of saporin-6.

The three dimensional structure reveals the active site residues, Glu176, Arg179 and Trp208 of saporin-6 to be completely superimposable on those of other RIPs (21). However,
the residue Tyr72 shown to be responsible for the interaction with the target adenine assumes different side chain conformations among all analyzed RIPs.

The role of active site residues of ricin Tyr80, Tyr123, Glu177 and Arg180, equivalent to Tyr72, Tyr120, Glu176 and Arg179 in saporin, has been studied earlier (22, 35-37). Tyr80 in ricin has been shown to make firmest physical contact with adenine and is more crucial to substrate recognition than Tyr123 (22). In ricin although Glu177 is proposed to be essential for transition state stabilization, its substitution with alanine facilitates the nearby Glu208 to move into the active site and fulfill a role similar to Glu177 (35,36). The complete loss of activity of saporin-6 mutant Y72A and a partial loss in activity of Y120A indicate a similar mechanism of action in saporin-6 and ricin. Compared to saporin-6 a 20-fold reduction was observed in the activity of E176A mutant. It appears that in the mutant E176A, Glu205 occupies the position of Glu176, however, carboxylate of Glu205 in the saporin-6 mutant E176A may provide less stabilization to the oxycarbonium ion transition state than Glu176. The mutation of Arg179 to alanine reduced the protein synthesis inhibitory activity of saporin-6 by 200-fold. Arg180, corresponding to Arg179 of saporin-6, has been shown in ricin to lie parallel to the conserved Trp211 residue, Trp208 in saporin-6, and make strong hydrogen bonds with O78 of the protein backbone, Glu177 and an active site water which may be involved in the RNA N-glycosidase reaction (37). This conserved arginine may also bind to the phosphate backbone of the 28S rRNA substrate. A similar mechanism seems to be operative in saporin-6 also.

The mutation of the only tryptophan residue of saporin-6 to alanine did not affect the enzymatic activity of the protein. Studies on PAP and abrin have suggested that Trp208 and Trp198 respectively in these proteins, corresponding to Trp208 in saporin-6, are crucial for structural integrity of these proteins (38,39).
Tyr16 and Arg24 are two of the nine invariant residues outside of the active site conserved among various RIPs (33). While mutation at Tyr16 to alanine resulted in complete loss of activity, mutating Arg24 did not have any effect on the enzymatic activity of saporin-6. Studies with trichosanthin (TCS) have suggested that the residues Y14 and R22, corresponding to Y16 and R24 of saporin-6, interact with the residues on the adjacent helix, which contains the active site residues Glu160 (Glu176 of saporin-6), and Arg163 (Arg179 of saporin-6) (40). The mutation of Tyr14 to Phe resulted in only 5-fold decrease in activity (40). In ricin A-chain, deletion of residues 21-23, thereby deleting Tyr21 equivalent to Tyr16 in saporin-6, did not affect the functional activity of the protein (41). These observations suggest that conserved Tyr at this position is not absolutely essential for the activity of RIPs. However, mutation of Y16 in saporin-6 to alanine resulted in complete loss of the RNA N-glycosidase activity.

**Cytotoxic activity of saporin-6 mutants**

A variety of cancer cell lines were treated with different concentrations of saporin-6 or the mutants and protein synthesis inhibition in the cells was taken as the measure of cytotoxicity. Saporin-6 caused a dose-dependent toxicity to all the cell lines. J774A.1 was the most sensitive cell line followed by L929, HUT 102 and U937 (Table 4). On J774A.1 cells, the mutant R24A had toxicity similar to that of saporin-6 and the mutant E176A had about 15-fold reduced activity (Table 4). Mutants W208A and Y16A although were toxic to J774A.1 cells, compared to saporin-6 they had about 200-fold lower activity (Table 4). Mutants Y120A and R179A manifested an extremely poor cytotoxicity on J774A.1 cells (Table 4). The mutant Y72A was totally non-toxic to J774A.1 cells. A similar pattern of cytotoxicity was observed with all the mutants, on the other three cell lines tested, however, the extent of toxicity and the fold difference between saporin and various mutants varied with
different cell lines (Table 4). The effect of various mutants on J774A.1 cells closely matched with that on U937 cells (Table 4).

The protein synthesis inhibitory activity of all the mutants correlated well with their ability to release the Endo-fragment. A comparison of the cytotoxicity and enzymatic activities, in vitro translation inhibition and production of the Endo-fragment, revealed the two activities for mutants R24A, Y72A, Y120A, E176A and R179A to be in complete correlation, i.e., the mutants having in vitro protein synthesis inhibitory activity and the Endo-fragment release activity demonstrated cytotoxicity. The extent of cytotoxicity corresponded quantitatively to the enzymatic activities of the mutants. Saporin-6 and the mutant R24A showed similar enzymatic activities and similar cytotoxicities on all the cell lines. Y72A did not have any enzymatic activity and lacked cytotoxicity as well. E176A showed 1-25% cytotoxic activity of the native toxin (Table 4), consistent with its partial enzymatic activity. Y120A also showed partial cytotoxicity agreeing with its reduced enzymatic activity. R179A was found to be partially active in the Endo-fragment assay, and had significantly less cytotoxicity. Interestingly, for the mutants Y16A and W208A there was no correlation between their cytotoxic activity and enzymatic activities. The activity of the mutant W208A was found to be similar to that of saporin-6 in both in vitro protein synthesis inhibitory assay and Endo-fragment release assay, however, its cytotoxicity was found to be significantly lower than the native toxin. On the other hand, the mutant Y16A was found to be inactive in both protein synthesis inhibition assay and Endo-fragment release assay, and yet it showed partial cytotoxicity on all the cell lines tested. Thus, it appeared that cytotoxicity of saporin-6 is not solely the consequence of its RNA N-glycosidase activity, and other activities or factors may also be involved.
Effect of saporin-6 and its mutants on genomic DNA

For almost two decades it was largely assumed that RIPs act only on rRNA within ribosomes (3,42). Recently, however, all plant RIPs and Shiga toxin have been shown to remove several adenine residues from naked RNAs and from DNA in vitro (9,43,44). Some RIPs have been shown to possess direct DNase activity also (24,45). It has been proposed to replace the term ribosome-inactivating protein with polynucleotide:adenosine glycosidase (9). This has raised great interest in the study of mechanism of action of RIPs on intact cells, to investigate the potential contribution of various activities of RIPs to their cytotoxicity.

The effect of saporin-6 was monitored on the genomic DNA of U937 cells in an attempt to ascertain the contribution of this activity to the cytotoxicity. U937 cells were incubated with 0.1 µM and 1 µM of saporin-6 for various time intervals (Figure 4A). In the presence of 1 µM saporin-6, significant DNA fragmentation started within 24 hours of incubation and became more pronounced at 36 and 48 hours. The control, where no toxin was added, showed a high molecular weight genomic DNA band throughout the course of study (Figure 4A). With 0.1 µM toxin there was no laddering after 24 hours, however, in 36 hours faint small molecular weight bands could be seen and a further 12 hours incubation resulted in a laddered appearance of the genomic DNA (Figure 4A). In order to investigate the effect of mutations of active site residues and invariant residues in saporin-6 on its ability to fragment genomic DNA, U937 cells were incubated in the presence of 1 µM of mutant proteins for 48 hours. The genomic DNA was isolated and analysed on agarose gels. As shown in Figure 4B, the active site mutants Y72A, Y120A, E176A, R179A and W208A failed to affect the genomic DNA. The cells treated with these proteins showed a high molecular weight intact DNA band as seen in the control. However, with mutants Y16A and R24A fragmented DNA was obtained similar to that with the native saporin-6 (Figure 4B).
Table 5 summarizes the RNA N-glycosidase activity, cytotoxicity and genomic DNA fragmentation activity of saporin-6 mutants. The activity of all the mutants on genomic DNA did not correlate with their RNA N-glycosidase activity. The wild type saporin-6 and the mutant R24A showed similar RNA N-glycosidase activity, cytotoxicity and genomic DNA fragmentation activity. The mutant Y72A did not show any RNA N-glycosidase activity and cytotoxicity, and did not cause any genomic DNA fragmentation. The mutants Y120A, E176A and R179A had no DNA fragmentation activity, however, they showed partial RNA N-glycosidase activity and poor cytotoxic activity. The mutant W208A possessed full RNA N-glycosidase activity, had reduced cytotoxicity but failed to affect the genomic DNA. The mutant Y16A did not show any RNA N-glycosidase activity, possessed reduced cytotoxicity similar to that of W208A, however, it caused the genomic DNA fragmentation comparable to native saporin-6. These results clearly demonstrate that saporin-6 possesses two independent activities, namely RNA N-glycosidase activity and genomic DNA fragmentation activity, and both are required for the cytotoxicity of the toxin.

Role of RNA N-glycosidase and genomic DNA fragmentation activity in saporin-6 cytotoxicity

The cytotoxicity data of various mutants indicated that for complete cytotoxic activity of saporin-6 both RNA N-glycosidase and genomic DNA fragmentation activities are required (Table 5). Loss of either one of these activities resulted in a reduction or loss of the cytotoxic activity. The mutants Y16A and W208A, each possessed one of the two activities, DNA fragmentation activity and rRNA N-glycosidase activity respectively, and showed similar but much reduced cytotoxicities (Table 5).

In order to further confirm the contribution of the two enzymatic activities of saporin-6 to its cytotoxic activity, U937 and J774 A.1 cells were treated with various concentrations of Y16A, W208A, and an equimolar mixture of the two proteins. As
mentioned before, Y16A and W208A showed a comparable, about 6-fold less cytotoxic activity than saporin-6 on U937 cells, and on J774 A.1 cells they had 230- and 125-fold less activity (Table 6). An equimolar mixture of Y16A and W208A, resulted in the cytotoxicity very similar to that of saporin-6 on U937 cells, whereas the mixture had a 5- and 3-fold improved cytotoxicity compared to that of the individual mutant proteins (Table 6). The cytotoxicity of saporin-6, therefore, appears to be a combined effect of its RNA N-glycosidase and DNA fragmentation activities.

**Intracellular localization of saporin-6**

To investigate if saporin translocates to nucleus to degrade DNA, J774A.1 cells were treated with iodinated saporin-6 for 3, 6, 9 and 16 hours and presence of radio-labelled protein was checked in the nuclear, cytosolic and membrane fractions. As shown in Figure 5, the concentration of saporin-6 increased in cytosol up to 6 hours, decreased gradually and became negligible by 16 hours. The membrane fraction did not show any significant amount of protein at any time point. The concentration of the protein in nuclear fraction was found to be comparable to that in the cytosolic fraction up to 9 hours, however, by 16 hours concomitant with the decrease in cytosol, most of the labeled saporin-6 localized in the nucleus (Figure 5). Apart from the intact ~30 kDa saporin-6 band, the nuclear fraction also showed some low molecular weight bands of ~17 and 25 kDa, seen earlier in the membrane fraction indicating onset of degradation of saporin-6 at longer incubation periods (Figure 5). The study shows that after internalization initially the protein stays in the cytosol and later migrates to the nucleus. Studies with Shiga toxin have shown that internalized Shiga toxin-I reaches nuclear envelop and the cells treated with Shiga toxin show the toxin predominantly in the nuclear fraction (46, 47). Saporin-6 does not possess any apparent nuclear localization signal. It appears that the primary target inside the cell is rRNA and activity on genomic DNA is a late event in cytotoxicity.
Recent studies on ricin and Shiga toxin suggest that these RIPs can damage nuclear DNA in whole cells by means that are not secondary to ribosome inactivation (48). Shiga toxin has been shown to release adenine from DNA by its RNA N-glycosidase activity that leads to spontaneous break of sugar-phosphate backbone whereas saporin-6, dianthin-30 and gelonin have been reported to manifest direct DNase like activity on plasmid DNA (24,49). The key residues involved in the catalytic activity of saporin appear to be functionally similar to homologous residues in ricin A and other RIPs. The current study demonstrates that the DNA fragmentation observed is not entirely dependent on the RNA N-glycosidase activity. The comparison of activities of saporin-6 mutants also suggests that there is a considerable overlap between the residues required for RNA N-glycosidase activity and genomic DNA fragmentation activity. Y72, Y120, E176 and R179 are important for the activity on both DNA and rRNA. W208 appears to be required only for the genomic DNA fragmentation activity. Substitution of Y16 abolished the activity of protein on rRNA, however it did not affect the genomic DNA fragmentation activity. The residue R24 can be dispensed with for both the activities of saporin-6. The crystal structure of saporin-6 and superimposition of the structure with other RIPs has suggested that the loop between its $\beta 7$ and $\beta 8$ strands is particularly short and it makes active site more accessible to various different adenine-containing substrates (21). Therefore, it appears that saporin-6 binds to DNA and rRNA through the same active site, however, the local positioning of the two substrates could be different enabling different residues to interact. The cytotoxic activity appears to require both the activities and loss or reduction of one results in a loss or reduction in the cytotoxic activity of saporin.

In conclusion, we have shown that saporin-6 possesses two catalytic activities namely RNA N-glycosidase and genomic DNA fragmentation activity. The cytotoxic activity of saporin-6 is governed by both these activities, which share considerable overlap in terms of
amino acid residue requirement. Tyr72 and Arg179 are absolutely indispensable for RNA N-glycosidase as well as genomic DNA fragmentation activity, whereas Arg24 does not seem to be playing any role in any of these activities of saporin-6. Tyr120, Glu176 and Trp208 play an important role in DNA fragmentation activity. However, Trp208 could be dispensed with completely, and Tyr120 and Glu176 partially, for the RNA N-glycosidase activity. Tyr16 appears to be required for the maintenance of a conformation essential for RNA N-glycosidase activity.
ACKNOWLEDGMENTS

This work was supported by grants to the National Institute of Immunology from the Department of Biotechnology, Government of India. Shveta Bagga is a Senior Research Fellow of the Council of Scientific and Industrial Research, India.
REFERENCES:


LEGENDS TO FIGURES

Figure 1. SDS-PAGE and Western blot analysis of saporin-6 and the mutants.
The proteins were expressed in BL21 (λDE3) cells of E. coli and purified from inclusion
cells by cation-exchange and gel filtration chromatography. The recombinant proteins were
analysed by 12.5% SDS-PAGE under reducing conditions followed by Coomassie blue
staining (A). Western blot analysis of the mutants was done using a polyclonal antibody
raised against saporin-6 (B). The different lanes in panel B correspond to the same proteins as
in panel A.

Figure 2. CD spectral analysis of saporin-6 and the mutants. CD spectra were
recorded in far-UV region (200-250 nm) at 25°C. and the spectra are presented as the mean
residue ellipticity. A. Saporin-6 (———), Y72A (······), and Y120A (--------------------); B.
Saporin-6 (———), E176A (······), R179A (--------------------), and W208A (······); C.
Saporin-6 (———), Y16A (······), and R24A (--------------------).

Figure 3. Specific RNA N-glycosidase activity of saporin-6 and the mutants on
28S rRNA. Rabbit reticulocyte lysate was treated with various proteins at indicated
concentrations. The reaction was carried at 30°C for 30 minutes followed by termination of
reaction using 0.4 % SDS. Total RNA was extracted and half of it was treated with aniline
acetate at 60°C for 3 minutes. Following vacuum drying the aniline, treated (+) and untreated
(-) RNA was resolved by electrophoresis on 2% agarose gel.

Figure 4. Genomic DNA laddering by saporin-6 and the mutants. DNA was
isolated from equal number of U937 cells treated with saporin-6 or the mutant. The samples
were run on 1.5% agarose gel and visualized by staining with ethidium bromide. A. Genomic
DNA profile after treatment of U937 cells with saporin-6 for indicated time and
concentration; B. Genomic DNA isolated from U937 cells after treatment with 1 µM mutant
protein for 48 hours.
Figure 5. Intracellular localization of radio-labeled saporin-6. J774A.1 cells were incubated with radio-labeled saporin-6 for indicated time points, cells were washed and incubated further for one hour. The cells were homogenized and separated into the nuclear, cytosolic and membrane fractions by ultracentrifugation. Proteins were precipitated from the cytosolic and membrane fractions using 20% TCA, the samples were analyzed by 12.5% SDS-PAGE followed by autoradiography. H, total homogenate; N, nuclear fraction; C, cytosol; and M, membrane fraction.
Table 1

The letters in small case represent the nucleotides mutated. The underlined sequence denotes Hind III site created in the mutant through the primer for screening.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Mutant</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>JKB54</td>
<td>Y16A</td>
<td>5’ ATCCACAAAAAGATGAagcTTGACCCGCGGTCGG 3’</td>
</tr>
<tr>
<td>JKB55</td>
<td>R24A</td>
<td>5’ ATCCTTTACGTGTTagcGATTTTATCCACAAA 3’</td>
</tr>
<tr>
<td>JKB56</td>
<td>Y72A</td>
<td>5’ AAGATACGCGACCACagcCAAGTTATCGCGTTT 3’</td>
</tr>
<tr>
<td>JKB57</td>
<td>Y120A</td>
<td>5’ CTTTTTCGATTGACTGagcATCTTCTGTGTATTC 3’</td>
</tr>
<tr>
<td>JKB58</td>
<td>E176A</td>
<td>5’ CCTAAATCGTGCTACagcTGCTGTCATTTGAAT 3’</td>
</tr>
<tr>
<td>JKB59</td>
<td>R179A</td>
<td>5’ TTGAATGTACCTAAAgcTGCTACCTCAGCTGT 3’</td>
</tr>
<tr>
<td>JKB60</td>
<td>W208A</td>
<td>5’ CGTAGAAATCTTACGagcGCTGACTTCAAATTG 3’</td>
</tr>
</tbody>
</table>
Table 2

CD spectra of the proteins were acquired at a scan speed of 50 nm/minute with a sensitivity of 50 mdegree and a response time of 1 second. Yang’s reference parameters were used to perform secondary structure analysis from CD measurements.

<table>
<thead>
<tr>
<th>Protein</th>
<th>α-Helix</th>
<th>β-sheet</th>
<th>Turn</th>
<th>Random</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saporin-6</td>
<td>14.3</td>
<td>42.8</td>
<td>13.4</td>
<td>29.5</td>
</tr>
<tr>
<td>Y16A</td>
<td>20.7</td>
<td>21.6</td>
<td>20.6</td>
<td>37.1</td>
</tr>
<tr>
<td>R24A</td>
<td>12.8</td>
<td>41.1</td>
<td>13.5</td>
<td>32.6</td>
</tr>
<tr>
<td>Y72A</td>
<td>17.8</td>
<td>29.4</td>
<td>20.4</td>
<td>32.3</td>
</tr>
<tr>
<td>Y120A</td>
<td>17.0</td>
<td>32.2</td>
<td>19.5</td>
<td>31.3</td>
</tr>
<tr>
<td>E176A</td>
<td>15.7</td>
<td>33.8</td>
<td>17.8</td>
<td>32.7</td>
</tr>
<tr>
<td>R179A</td>
<td>19.1</td>
<td>25.7</td>
<td>21.5</td>
<td>33.7</td>
</tr>
<tr>
<td>W208A</td>
<td>19.5</td>
<td>23.0</td>
<td>20.7</td>
<td>36.8</td>
</tr>
</tbody>
</table>
Table 3

Rabbit reticulocyte lysate was treated with various concentrations of saporin-6 or the mutant at 30°C for one hour. ID$_{50}$ values refer to the concentration of toxin causing 50% inhibition of protein synthesis.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ID$_{50}$ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saporin-6</td>
<td>4.5</td>
</tr>
<tr>
<td>Y16A</td>
<td>&gt;1000.0</td>
</tr>
<tr>
<td>R24A</td>
<td>5.8</td>
</tr>
<tr>
<td>Y72A</td>
<td>&gt;1000.0</td>
</tr>
<tr>
<td>Y120A</td>
<td>480.0</td>
</tr>
<tr>
<td>E176A</td>
<td>100.0</td>
</tr>
<tr>
<td>R179A</td>
<td>1000.0</td>
</tr>
<tr>
<td>W208A</td>
<td>6.0</td>
</tr>
</tbody>
</table>
Table 4

Cells were treated with different concentrations of saporin-6 or the mutants for 36 hours. Incorporation of [$^3$H]-leucine in the newly synthesized proteins was measured. ID50 refers to the amount of toxin required to inhibit cellular protein synthesis by 50%. The numbers in parenthesis indicate percentage activity compared to the native toxin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ID$_{50}$ (µg/ml)</th>
<th>J774A.1</th>
<th>HUT102</th>
<th>L929</th>
<th>U937</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saporin</td>
<td>0.02 (100)</td>
<td>3.0 (100)</td>
<td>0.6 (100)</td>
<td>4.5 (100)</td>
<td></td>
</tr>
<tr>
<td>Y16A</td>
<td>4.00 (0.50)</td>
<td>27.0 (11)</td>
<td>37.0 (1.6)</td>
<td>32.0 (14)</td>
<td></td>
</tr>
<tr>
<td>R24A</td>
<td>0.02 (100)</td>
<td>1.4 (200)</td>
<td>1.8 (33.4)</td>
<td>6.4 (70)</td>
<td></td>
</tr>
<tr>
<td>Y72A</td>
<td>&gt;100 (&lt;0.02)</td>
<td>&gt;100.0 (&lt;3)</td>
<td>100.0 (0.6)</td>
<td>86.0 (5)</td>
<td></td>
</tr>
<tr>
<td>Y120A</td>
<td>100.00 (0.02)</td>
<td>&gt;100.0 (&lt;3)</td>
<td>50.0 (1.2)</td>
<td>28.0 (16)</td>
<td></td>
</tr>
<tr>
<td>E176A</td>
<td>0.30 (6.0)</td>
<td>12.0 (25)</td>
<td>55.0 (1.1)</td>
<td>45.0 (10)</td>
<td></td>
</tr>
<tr>
<td>R179A</td>
<td>18.00 (0.10)</td>
<td>&gt;100.0 (&lt;3)</td>
<td>&gt;100.0 (&lt;0.6)</td>
<td>&gt;100.0 (4.5)</td>
<td></td>
</tr>
<tr>
<td>W208A</td>
<td>3.00 (0.70)</td>
<td>25.0 (12.0)</td>
<td>40.0 (1.5)</td>
<td>21.0 (21.5)</td>
<td></td>
</tr>
</tbody>
</table>
Table 5

Translation inhibition is expressed as percent activity of the native toxin. Cytotoxic activity is shown for U937 cells. P. Active refers to partially active as compared to the native toxin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Translation inhibition</th>
<th>Endo-fragment release</th>
<th>Cytotoxicity</th>
<th>DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saporin-6</td>
<td>100.00</td>
<td>Active</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Y16A</td>
<td>&lt;0.45</td>
<td>Inactive</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>R24A</td>
<td>78.00</td>
<td>Active</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Y72A</td>
<td>&lt;0.45</td>
<td>Inactive</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Y120A</td>
<td>1.00</td>
<td>P. Active</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>E176A</td>
<td>4.50</td>
<td>P. Active</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>R179A</td>
<td>0.45</td>
<td>P. Active</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>W208A</td>
<td>75.00</td>
<td>Active</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 6

U937 and J774A.1 cells were treated with various concentrations of saporin, Y16A, W208A, and an equimolar mixture of Y16A and W208A for 36 hours. Incorporation of [3H]-leucine was measured in the newly synthesized protein. ID_{50} refers to the concentration of toxin causing 50% inhibition of protein synthesis compared to controls where no toxin was added.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ID_{50} (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>U937</td>
</tr>
<tr>
<td>Saporin</td>
<td>5.2</td>
</tr>
<tr>
<td>W208A</td>
<td>32.0</td>
</tr>
<tr>
<td>Y16A</td>
<td>32.0</td>
</tr>
<tr>
<td>W208A+Y16A</td>
<td>9.0</td>
</tr>
</tbody>
</table>
A. SDS-PAGE

B. Western-blot
Figure 2

(A) (B) (C)
Figure 3

Protein, ng/ml

<table>
<thead>
<tr>
<th>Protein</th>
<th>0</th>
<th>40</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aniline</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

28S rRNA

18S rRNA

Endo-fragment

Saporin-6

Y72A

Y16A

W208A

R24A

Y120A

E176A

R179A

5' fragment

5' fragment
Figure 4

A.

<table>
<thead>
<tr>
<th>Protein, µM</th>
<th>24 hours</th>
<th>36 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

B. 

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3054</td>
</tr>
<tr>
<td>Saporin-6</td>
<td>2036</td>
</tr>
<tr>
<td>Y16A</td>
<td>1636</td>
</tr>
<tr>
<td>R24A</td>
<td>1018</td>
</tr>
<tr>
<td>Y72A</td>
<td>517</td>
</tr>
<tr>
<td>Y120A</td>
<td>396</td>
</tr>
<tr>
<td>E176A</td>
<td>220</td>
</tr>
<tr>
<td>R179A</td>
<td>208A</td>
</tr>
</tbody>
</table>
The cytotoxic activity of ribosome-inactivating protein saporin-6 is attributed to rRNA N-glycosidase and internucleosomal DNA fragmentation
Shveta Bagga, Divya Seth and Janendra K. Batra

J. Biol. Chem. published online December 3, 2002

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