THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 7, Issue of February 14, pp. 4813–4820, 2003

Printed in U.S.A.

The Cytotoxic Activity of Ribosome-inactivating Protein Saporin-6 Is Attributed to Its rRNA *N*-Glycosidase and Internucleosomal DNA Fragmentation Activities*

Received for publication, July 23, 2002, and in revised form, November 22, 2002
Published, JBC Papers in Press, December 3, 2002, DOI 10.1074/jbc.M207389200

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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 28 S 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, Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208}, and two invariant residues, Tyr^{16} and Arg^{24}, 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 Tyr^{16}, Tyr^{72}, and Arg^{179} are absolutely critical; Tyr^{120} and Glu^{176} can be partially dispensed with, whereas Trp^{208} and Arg^{24} do not appear to be involved in this activity. The residues Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208} were found to be essential for the genomic DNA fragmentation activity, whereas residues Tyr^{16} and Arg^{24} 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.

Ribosome-inactivating proteins (RIPs)[1] 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, whereas type II RIPs consist of two chains, A and B, linked by a disulfide bond (1). Type I RIPs (*e.g.* saporin, trichosanthin, and pokeweed antiviral protein) and A-chain of type II RIPs (*e.g.* ricin and abrin) inhibit protein synthesis by removing a specific adenine from 28 S rRNA of eukaryotic ribosomes. The former also removes an equivalent adenine residue, A^{2660}, from 23 S rRNA of *Escherichia coli* ribosomes (2). In both cases, the site of action is located in a highly conserved, purine-rich stem and loop structure of rRNA termed 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 toward various cell lines, their different requirements for cofactors, and variations in the minimal structure of the adenine-containing loop that they can attack point to their substantial diversity (6, 7). The study of molecules that 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 nonribosomal 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 pokeweed antiviral protein cleaves the double-stranded supercoiled DNA using the same active site required to depurinate rRNA and that momordin 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 Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208} 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 Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208} were mutated to alanine, and the activities of the mutants were compared with 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 the ricin sequence. An analysis of ricin-A chain structure showed that a central α-he-

* This work was supported by grants from the Department of Biotechnology, Government of India (to the National Institute of Immunology). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: RIP, ribosome-inactivating protein; FCS, fetal calf serum.

TABLE I
Sequence of primers used for mutagenesis of residues in saporin-6
The letters in lowercase represent the nucleotides mutated. The underlined sequence denotes the *Hin*dIII site created in the mutant through the primer for screening.

Primer	Mutant	Sequence
JKB54	Y16A	5′-ATCCACAAAAGATGAagcTTGACCCGCGGTCGG-3′
JKB55	R24A	5′-ATCCTTTACGTTGTTagcGATTTTATCCACAAA-3′
JKB56	Y72A	5′-AAGATACGCGACCACagcCAAGTTATCGCGTTT-3′
JKB57	Y120A	5′-CTTTTCGATTGACTGagcATCTTCTGTGTATTC-3′
JKB58	E176A	5′-CCTAAATCGTGCTACagcTGCTGTCATTTGAAT-3′
JKB59	R179A	5′-TTGAATGTACCTAAAagcTGCTACCTCAGCTGT-3′
JKB60	W208A	5′-CGTAGAAATCTTACGagcGCTGACTTCAAATTG-3′

lix is bent near its C terminus, and this bending allows the catalytic residues, Glu^{177} and Arg^{180}, to reach the solvent of the active site cleft (23). This important helix bending disrupts the normal α-helical bonding pattern. However, the resulting structure is stabilized by new hydrogen bonds to the side chains of Tyr^{21} and Arg^{29}. 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, Tyr^{16} and Arg^{24}, 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 Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208} to that for alanine. Similarly, the codons for the invariant residues Tyr^{16} and Arg^{24} were also mutated to that for alanine. All of the mutations were carried out by oligonucleotide-mediated site-directed mutagenesis (25). Uracil-containing DNA template was prepared by infecting the CJ236 strain of *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 I. The primer extension products were transformed into *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 *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 of the mutants were found to accumulate in the form of inclusion bodies, and they were purified using the procedure described by Buchner *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-fold dilution of the protein in a refolding buffer containing L-arginine and oxidized glutathione. After incubating at 10 °C for 48 h, 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/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 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 28 S 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 0.5 h 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 min. Total RNA was isolated using Trizol reagent as per the manufacturer's 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 min, 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% bromphenol blue was added. The samples were heated at 65 °C for 5 min 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 the assay was 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 of [^{3}H]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 bovine serum albumin. The endogenous globin mRNA in the reticulocyte lysate was used for translation. The reaction was carried out at 30 °C for 1 h and stopped by adding 0.25 ml of 1 N NaOH containing 0.2% H_2O_2, followed by an incubation at 37 °C for 10 min. The proteins were precipitated with 15% trichloroacetic acid on ice for 30 min and harvested on 26-mm glass fiber 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-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 5×10^3 cells/well in a 96-well plate in 0.2 ml of RPMI/Dulbecco's modified Eagle's medium containing 10% FCS for 16 h. The medium was replaced with 0.2 ml of 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 in Dulbecco's PBS for 34 h followed by labeling with 0.75 μCi of [^{3}H]leucine/well for 2 h. The cells after freezing and thawing were harvested on filtermats using a 96-well plate automated harvester, and the filters were counted using

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 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. 5×10^5 cells were cultured in RPMI containing 10% FCS in the presence of different concentrations of proteins. After the 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 h. The DNA was extracted with phenol/chloroform and precipitated with isopropyl alcohol. 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 the 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 of $Na^{125}I$ were added to the iodogen-coated tube. The tube was tapped at room temperature for 10 min followed by the addition of 50 μl of 0.2% KI. The labeled protein was purified using PD-10 column (Amersham Biosciences) presaturated with bovine serum albumin.

J774A.1 cells, 6×10^6 were seeded in 6 ml of Dulbecco's modified Eagle's medium containing 10% FCS in a T-25 tissue culture flask and incubated at 37 °C for 16 h. The medium was replaced with 1 ml of Dulbecco's modified Eagle's medium 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 1 h 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 of buffer containing 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin and incubated on ice for 10 min. The cells were centrifuged at 3000 × *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 min at 3000 × *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 $MgCl_2$ was added, and the mixture was centrifuged at 100,000 × *g* for 30 min 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 of PBS. The proteins in various fractions were precipitated with 1 ml of 20% trichloroacetic acid. The precipitated proteins were boiled in SDS loading dye for 3 min 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 Tyr^{72}, Tyr^{120}, Glu^{176}, Arg^{179}, and Trp^{208}, 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, Tyr^{16} and Arg^{24}, 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 overexpressed 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 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 (Fig. 1). On Western blots, all of the mutants reacted equally well with a polyclonal antibody raised against saporin-6 (Fig. 1).

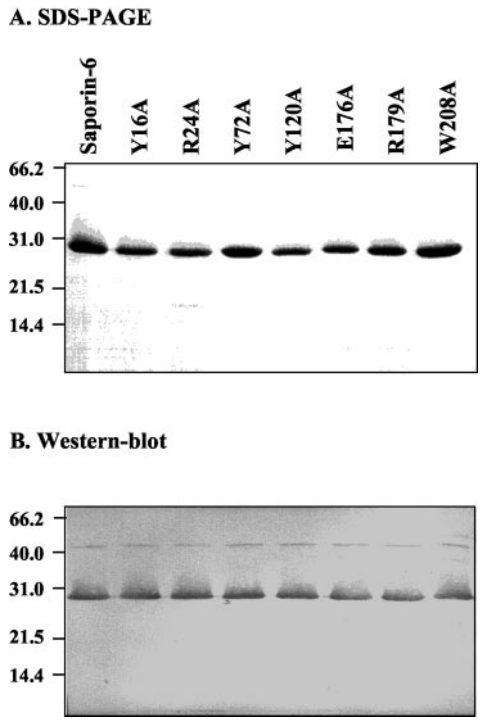

FIG. 1. **SDS-PAGE and Western blot analysis of saporin-6 and the mutants.** The proteins were expressed in BL21 (λDE3) cells of *E. coli* and purified from inclusion bodies by cation exchange and gel filtration chromatography. The recombinant proteins were analyzed by 12.5% SDS-PAGE under reducing conditions, 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 *B* correspond to the same proteins as in *A*.

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 α + β structure (Fig. 2). The substitution of active site residues Tyr^{72}, Glu^{176}, and Trp^{208} 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 (Fig. 2, *A* and *B*). The replacement of the invariant residue Tyr^{16} with alanine resulted in a significant shift in the CD spectra (Fig. 2*C*). The α-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 β-sheet content of these mutants compared with the native toxin (Table II). The mutant R24A appeared to be similarly folded as the native toxin (Fig. 2*C*). The quantitative values of various secondary structure elements of R24A were found to be similar to saporin-6 (Table II).

RNA N-Glycosidase Activity of Saporin-6 Mutants—The effect of substitution of various active site and invariant residues with alanine on the specific RNA *N*-glycosidase activity of saporin-6 was evaluated. 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 Fig. 3, aniline treatment of saporin-6-treated RNA samples resulted in the release of the classic 390-base Endo-fragment. Whereas saporin-6 produced the endofragment at as low as 40 ng/ml, the mutants Y72A and Y16A failed to affect rRNA even up to 1000 ng/ml (Fig. 3). With the mutants Y120A, E176A, and R179A, a faint endofragment was seen at 1000 ng/ml in the aniline-treated samples (Fig. 3).

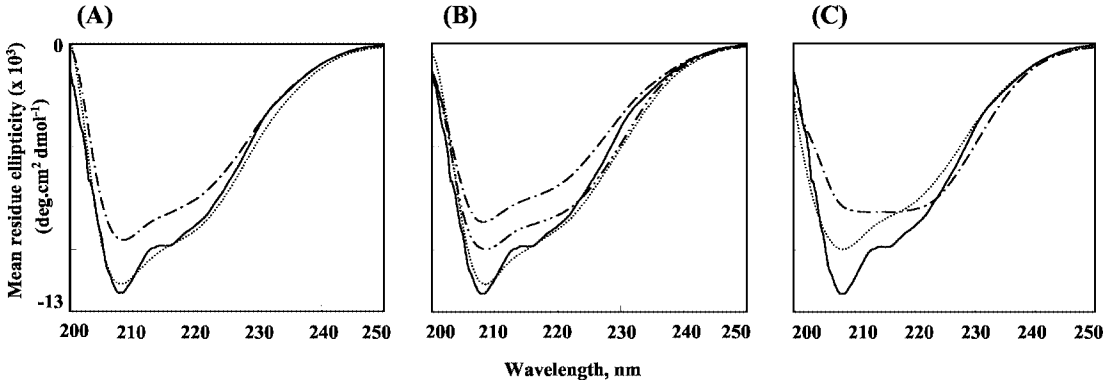

FIG. 2. **CD spectral analysis of saporin-6 and the mutants.** CD spectra were recorded in the far-UV region (200–250 nm) at 25 °C, and the spectra are presented as the mean residue ellipticity. *A*, saporin-6 (*solid line*), Y72A (*dotted and dashed line*), and Y120A (*dotted line*). *B*, saporin-6 (*solid line*), E176A (*dotted and dashed lines*), R179A (*dotted line*), and W208A (*dashes and double dots*). *C*, saporin-6 (*solid line*), Y16A (*dotted and dashed line*), and R24A (*dotted line*).

TABLE II
Secondary structure analysis of saporin-6 and the mutants

CD spectra of the proteins were acquired at a scan speed of 50 nm/min with a senstivity of 50 millidegrees and a response time of 1 s. Yang's reference parameters were used to perform secondary structure analysis from CD measurements.

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

Substitution of Trp^{208} or Arg^{24} with alanine did not have any effect on the RNA *N*-glycosidase activity of saporin-6, since the mutants W208A and R24A released the Endo-fragment from 28 S rRNA at concentrations similar to that of the native toxin (Fig. 3). The intensity of endofragment increased with increasing concentrations of the toxin, indicating a dose-dependent response. The decrease in size of 28 S rRNA upon release of the 390-base fragment was also apparent in aniline-treated samples of the mutants W208A and R24A (Fig. 3).

The release of Endo-fragment from 28 S rRNA 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}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_{50} of 4.5 ng/ml (Table III). The mutations of Trp^{208} and Arg^{24} 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_{50} values of 6.0 and 5.8 ng/ml (Table III). The substitution of invariant residue Tyr^{16} and the active site residue Tyr^{72} with alanine abolished the protein synthesis-inhibitory activity of the protein, and even in the presence of 1000 ng/ml Y16A or Y72A, there was no inhibition of protein synthesis (Table III). The mutants Y120A and E176A, however, caused protein synthesis inhibition but at a relatively higher concentration compared with the native toxin. The respective ID_{50} values of Y120A and E176A were 480 and 100 ng/ml, showing these mutants to be 100- and 20-fold less active than the native toxin (Table III). The mutant R179A showed an extremely poor protein synthesis inhibitory activity; its ID_{50} was found to be 200-fold less than the native toxin (Table III). 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, Tyr^{72} and Arg^{179} are absolutely essential for the RNA *N*-glycosidase activity of saporin-6. The residues Tyr^{120} and Glu^{176} can be partially dispensed with, whereas Trp^{208} and Arg^{24} are not required for the RNA *N*-glycosidase activity. The invariant residue Tyr^{16} also appears to be critical for the RNA *N*-glycosidase activity of saporin-6.

The three-dimensional structure reveals the active site residues, Glu^{176}, Arg^{179}, and Trp^{208}, of saporin-6 to be completely superimposable on those of other RIPs (21). However, the residue Tyr^{72}, 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 Tyr^{80}, Tyr^{123}, Glu^{177}, and Arg^{180}, equivalent to Tyr^{72}, Tyr^{120}, Glu^{176}, and Arg^{179} in saporin, has been studied earlier (22, 35–37). Tyr^{80} in ricin has been shown to make the firmest physical contact with adenine and is more crucial to substrate recognition than Tyr^{123} (22). In ricin, although Glu^{177} is proposed to be essential for transition state stabilization, its substitution with alanine facilitates the nearby Glu^{208} to move into the active site and fulfill a role similar to that of Glu^{177} (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 with saporin-6, a 20-fold reduction was observed in the activity of E176A mutant. It appears that in the mutant E176A, Glu^{205} occupies the position of Glu^{176}; however, carboxylate of Glu^{205} in the saporin-6 mutant E176A may provide less stabilization to the oxycarbonium ion transition state than Glu^{176}. The mutation of Arg^{179} to alanine reduced the protein synthesis-inhibitory activity of saporin-6 by 200-fold. Arg^{180}, corresponding to Arg^{179} of saporin-6, has been shown in ricin to lie parallel to the conserved Trp^{211} residue, Trp^{208}, in saporin-6 and make strong hydrogen bonds with O-78 of the protein backbone, Glu^{177} and an active site water that may be involved in the RNA *N*-glycosidase reaction (37). This conserved arginine may also bind to the phosphate backbone of the 28 S 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 pokeweed antiviral protein and abrin have suggested that Trp^{208} and Trp^{198}, respectively, in these proteins,

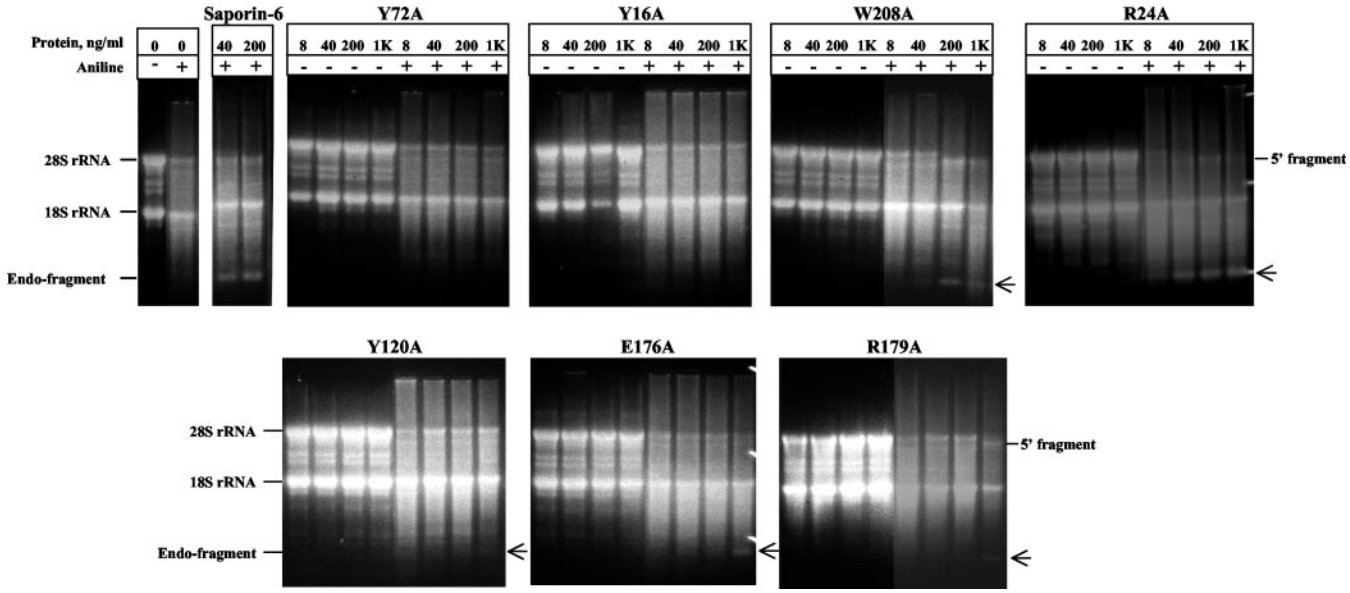

FIG. 3. **Specific RNA *N*-glycosidase activity of saporin-6 and the mutants on 28 S rRNA.** Rabbit reticulocyte lysate was treated with various proteins at the indicated concentrations. The reaction was carried at 30 °C for 30 min followed by termination of the reaction using 0.4% SDS. Total RNA was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

TABLE III
In Vitro protein synthesis inhibitory activity of saporin-6 and the mutants

Rabbit reticulocyte lysate was treated with various concentrations of saporin-6 or the mutant at 30 °C for 1 h. ID_{50} values refer to the concentration of toxin causing 50% inhibition of protein synthesis.

Protein	ID_{50}
	(ng/ml)
Saporin-6	4.5
Y16A	>1000.0
R24A	5.8
Y72A	>1000.0
Y120A	480.0
E176A	100.0
R179A	1000.0
W208A	6.0

corresponding to Trp^{208} in saporin-6, are crucial for structural integrity of these proteins (38, 39).

Tyr^{16} and Arg^{24} are two of the nine invariant residues outside of the active site conserved among various RIPs (33). Whereas mutation at Tyr^{16} to alanine resulted in complete loss of activity, mutating Arg^{24} did not have any effect on the enzymatic activity of saporin-6. Studies with trichosanthin have suggested that the residues Tyr^{14} and Arg^{22}, corresponding to Tyr^{16} and Arg^{24} of saporin-6, interact with the residues on the adjacent helix, which contains the active site residues Glu^{160} (Glu^{176} of saporin-6) and Arg^{163} (Arg^{179} of saporin-6) (40). The mutation of Tyr^{14} to Phe resulted in only a 5-fold decrease in activity (40). In ricin A-chain, deletion of residues 21–23 (thereby deleting Tyr^{21}, equivalent to Tyr^{16} 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 Tyr^{16} 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 of the cell lines. J774A.1 was the most sensitive cell line, followed by L929, HUT 102, and U937 (Table IV). 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 IV). Although mutants W208A and Y16A were toxic to J774A.1 cells, compared with saporin-6 they had about 200-fold lower activity (Table IV). Mutants Y120A and R179A manifested an extremely poor cytotoxicity on J774A.1 cells (Table IV). The mutant Y72A was totally nontoxic to J774A.1 cells. A similar pattern of cytotoxicity was observed with all of the mutants on the other three cell lines also, however, the extent of toxicity and the -fold difference between saporin and various mutants varied with different cell lines (Table IV). The effect of various mutants on J774A.1 cells closely matched with that on U937 cells (Table IV).

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 endofragment 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 of 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 IV), 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, yet it showed partial cytotoxicity on all

TABLE IV
Cytotoxic activity of saporin-6 and the mutants

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

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

of 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). There has been a proposal to replace the term "ribosome-inactivating protein" with "polynucleotide:adenosine glycosidase" (9). This has raised great interest in the study of the 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 and 1 μM of saporin-6 for various time intervals (Fig. 4*A*). In the presence of 1 μM saporin-6, significant DNA fragmentation started within 24 h of incubation and became more pronounced at 36 and 48 h. The control, where no toxin was added, showed a high molecular weight genomic DNA band throughout the course of study (Fig. 4*A*). With 0.1 μM toxin there was no laddering after 24 h; however, in 36 h faint small molecular weight bands could be seen, and a further 12-h incubation resulted in a laddered appearance of the genomic DNA (Fig. 4*A*). 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 mutant proteins for 48 h. The genomic DNA was isolated and analyzed on agarose gels. As shown in Fig. 4*B*, 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 (Fig. 4*B*).

Table V 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

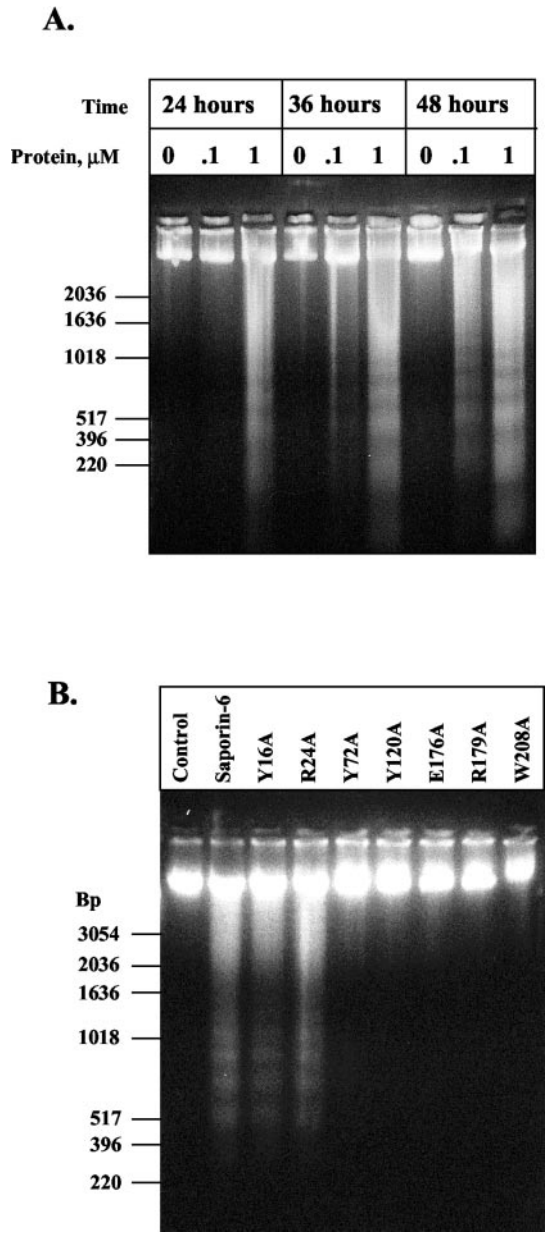

FIG. 4. **Genomic DNA laddering by saporin-6 and the mutants.** DNA was isolated from equal number of U937 cells treated with saporin-6 or the mutant. The samples were run on 1.5% agarose gel and visualized by staining with ethidium bromide. *A*, genomic DNA profile after treatment of U937 cells with saporin-6 for indicated time and concentration; *B*, genomic DNA isolated from U937 cells after treatment with 1 μM mutant protein for 48 h.

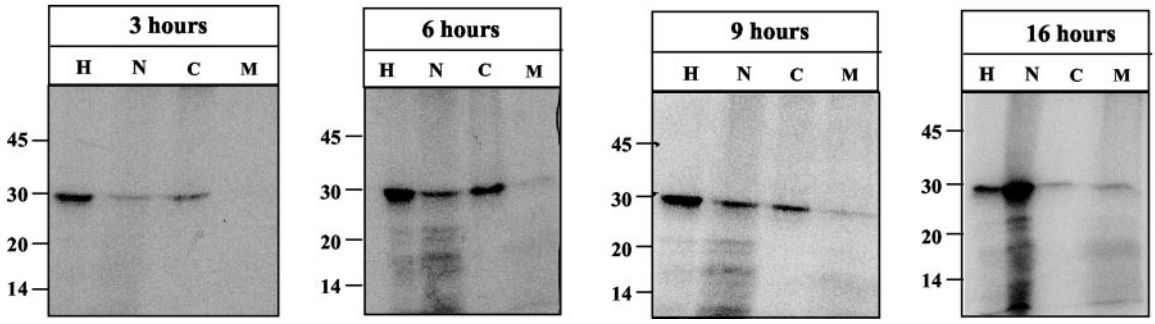

FIG. 5. **Intracellular localization of radiolabeled saporin-6.** J774A.1 cells were incubated with radiolabeled saporin-6 for the indicated time points, and cells were washed and incubated further for 1 h. The cells were homogenized and separated into the nuclear, cytosolic, and membrane fractions by ultracentrifugation. Proteins were precipitated from the cytosolic and membrane fractions using 20% trichloroacetic acid, the samples were analyzed by 12.5% SDS-PAGE followed by autoradiography. *H*, total homogenate; *N*, nuclear fraction; *C*, cytosol; *M*, membrane fraction.

TABLE V
Comparison of various activities of saporin-6 and the mutants

Translation inhibition is expressed as percentage of activity of the native toxin. Cytotoxic activity is shown for U937 cells. P. Active, partially active as compared with the native toxin.

Protein	Translation inhibition	Endo-fragment release	Cytotoxicity	DNA fragmentation
Saporin-6	100.00	Active	+++	+
Y16A	<0.45	Inactive	+	+
R24A	78.00	Active	+++	+
Y72A	<0.45	Inactive	−	−
Y120A	1.00	P. Active	+/−	−
E176A	4.50	P. Active	+	−
R179A	0.45	P. Active	+/−	−
W208A	75.00	Active	+	−

activity and had reduced cytotoxicity but failed to affect the genomic DNA. The mutant Y16A did not show any RNA *N*-glycosidase activity and possessed reduced cytotoxicity similar to that of W208A; however, it caused the genomic DNA fragmentation comparable with 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 V). 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 V).

In order to further confirm the contribution of the two enzymatic activities of saporin-6 to its cytotoxic activity, U937 and J774A.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 lower, cytotoxic activity than saporin-6 on U937 cells, and on J774A.1 cells they had 230- and 125-fold less activity (Table VI). 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 with that of the individual mutant proteins on J774A.1 cells (Table VI). 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 whether saporin translocates to nucleus to degrade DNA, J774A.1 cells were treated with iodinated saporin-6 for 3, 6, 9, and 16 h, and the presence of radiolabeled protein was checked in the nuclear, cytosolic, and membrane fractions. As shown in Fig. 5, the concentration of saporin-6 increased in cytosol up to 6 h, decreased gradually, and became negligible by 16 h. 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 with that in the cytosolic fraction up to 9 h; however, by 16 h, concomitant with the decrease in cytosol, most of the labeled saporin-6 localized in the nucleus (Fig. 5). Apart from the intact ~30-kDa saporin-6 band, the nuclear fraction also showed some low molecular mass bands of ~17 and 25 kDa, seen earlier in the membrane fraction, indicating onset of degradation of saporin-6 at longer incubation periods (Fig. 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 the nuclear envelope, 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

TABLE VI
Cytotoxic activity of Y16A, W208A, and saporin-6

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

Protein	ID_{50}	
	U937	J774A.1
	μg/ml	
Saporin	5.2	0.08
W208A	32.0	10.0
Y16A	32.0	19.0
W208A and Y16A	9.0	3.8

rRNA and that 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. Tyr^{72}, Tyr^{120}, Glu^{176}, and Arg^{179} are important for the activity on both DNA and rRNA. Trp^{208} appears to be required only for the genomic DNA fragmentation activity. Substitution of Tyr^{16} abolished the activity of protein on rRNA; however, it did not affect the genomic DNA fragmentation activity. The residue Arg^{24} can be dispensed with for both of 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 β_7 and β_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 of these activities, which share considerable overlap in terms of amino acid residue requirement. Tyr^{72} and Arg^{179} are absolutely indispensable for RNA *N*-glycosidase as well as genomic DNA fragmentation activity, whereas Arg^{24} does not seem to be playing any role in any of these activities of saporin-6. Tyr^{120}, Glu^{176}, and Trp^{208} play an important role in DNA fragmentation activity. However, Trp^{208} could be dispensed with completely, and Tyr^{120} and Glu^{176} partially, for the RNA *N*-glycosidase activity. Tyr^{16} appears to be required for the maintenance of a conformation essential for RNA *N*-glycosidase activity.

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