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

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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, Tyr72, Tyr120, Glu176, Arg179, and Trp208, and two invariant residues, Tyr16 and Arg24, 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 Tyr72, Tyr120, Glu176, Arg179, and Trp208 are absolutely critical; Tyr16 and Arg24 can be partially dispensed with, whereas Trp208 and Arg24 do not appear to be involved in this activity. The residues Tyr72, Tyr120, Glu176, Arg179, and Trp208 were found to be essential for the genomic DNA fragmentation activity, whereas residues Tyr16 and Arg24 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)† 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, A2660, 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 RNA 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 activity 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 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 Tyr72, Tyr120, Glu176, Arg179, and Trp208 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-
lix is bent near its C terminus, and this bending allows the catalytic residues, Glu<sup>177</sup> and Arg<sup>192</sup>, 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<sup>23</sup> and Arg<sup>25</sup>. 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<sup>23</sup> and Arg<sup>25</sup>, 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<sup>17</sup>, Tyr<sup>20</sup>, Glu<sup>176</sup>, Arg<sup>179</sup>, and Trp<sup>208</sup> to that for alanine. Similarly, the codons for the invariant residues Tyr<sup>15</sup> and Arg<sup>24</sup> in saporin-6, they were mutated to 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 (DE3) 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 with Triton X-100. The inclusion body pellet thus obtained was dissolved in guanidine hydrochloride and reduced by adding dithiothreitol. Renaturation was carried out by 100-fold dilution of the protein in a refolding buffer containing t-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.
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<sub>50</sub> values. The ID<sub>50</sub> 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<sup>5</sup> 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 proteasine 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<sup>125</sup>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<sup>6</sup> 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<sup>6</sup> 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<sub>2</sub>, 100 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 100,000 × g. The supernatant constituted the supernatant fraction, and the supernatant contained cytosol and membranes. The cytosol and pellet were centrifuged 3000 × 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<sup>12</sup>, Tyr<sup>220</sup>, Glu<sup>176</sup>, Arg<sup>179</sup>, and Trp<sup>208</sup>, 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<sup>16</sup> and Arg<sup>24</sup>, 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 on the specific RNA 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.

FIG. 1. SDS-PAGE and Western blot analysis of saporin-6 and the mutants. The proteins were expressed in BL21 (DE3) 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.

Cytotoxicity of Saporin-6

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<sup>12</sup>, Glu<sup>176</sup> and Trp<sup>208</sup> 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<sup>16</sup> with alanine resulted in a significant shift in the CD spectra (Fig. 2C). 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. 2C). 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).
Substitution of Trp208 or Arg24 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-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, Tyr72 and Arg179 are absolutely essential for the RNA N-glycosidase activity of saporin-6. The residues Tyr120 and Glu176 can be partially dispensed with, whereas Trp208 and Arg24 are not required for the RNA N-glycosidase activity. The invariant residue Tyr16 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 Arg179, equivalent to Tyr72, Tyr179, Glu176, and Arg179 in saporin, has been studied earlier (22, 35–37). Tyr80 in ricin has been shown to make the 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 that of 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 with saporin-6, a 20-fold reduction was observed in the activity of E176A mutant. It appears that in the mutant E176A, Glu208 occupies the position of Glu176; however, carboxylate of Glu208 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 O-78 of the protein backbone, Glu177, 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 Trp208 and Trp198, respectively, in these proteins,
Glu160 (Glu176 of saporin-6) and Arg 163 (Arg179 of saporin-6) on the adjacent helix, which contains the active site residues integrity of these proteins (38, 39). According to Tyr16 and Arg24 of saporin-6, interact with the residues of Tyr16 in saporin-6 to alanine resulted in complete loss of absolutely essential for the activity of RIPs. However, mutation observations suggest that conserved Tyr at this position is not did not affect the functional activity of the protein (41). These 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. 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 corresponding to Trp208 in saporin-6, are crucial for structural integrity of these proteins (38, 39).

**Tyr**16 and Arg24 are two of the nine invariant residues outside of the active site conserved among various RIPs (33). Whereas 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 have suggested that the residues Tyr14 and Arg22, corresponding to Tyr16 and Arg24 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 a 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 Tyr16 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).

**Table III**

<table>
<thead>
<tr>
<th>Protein</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt; (ng/ml)</th>
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<tbody>
<tr>
<td>Saporin-6</td>
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</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>

**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.
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 \( \mu \)g/ml of saporin-6 for various time intervals (Fig. 4A). In the presence of 1 \( \mu \)g/ml of 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. 4A). With 0.1 \( \mu \)g/ml of 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. 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 \( \mu \)g/ml of mutant proteins for 48 h. The genomic DNA was isolated and analyzed on agarose gels. As shown in Fig. 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 (Fig. 4B).

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 activity.

**TABLE IV**

Cytotoxic activity of saporin-6 and the mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>J74A.1</th>
<th>HUT102</th>
<th>L929</th>
<th>U937</th>
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<tr>
<td>Saporin</td>
<td>0.02 (100)</td>
<td>3.0 (100)</td>
<td>0.6 (100)</td>
<td>4.5 (100)</td>
</tr>
<tr>
<td>Y16A</td>
<td>4.00 (50)</td>
<td>27.0 (11)</td>
<td>37.0 (1.6)</td>
<td>32.0 (14)</td>
</tr>
<tr>
<td>R24A</td>
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<td>1.4 (200)</td>
<td>1.8 (33.4)</td>
<td>6.4 (70)</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>
</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 (1)</td>
</tr>
<tr>
<td>E176A</td>
<td>0.30 (6.0)</td>
<td>12.0 (25)</td>
<td>45.0 (10)</td>
<td></td>
</tr>
<tr>
<td>R179A</td>
<td>18.00 (1.0)</td>
<td>&gt;100.0 (&lt;3)</td>
<td>&gt;100.0 (&lt;0.6)</td>
<td>100.0 (4.5)</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>
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</tbody>
</table>

**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 mutants. 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 \( \mu \)g/ml of mutant protein for 48 h.
were analyzed by 12.5% SDS-PAGE followed by autoradiography. Proteins were precipitated from the cytosolic and membrane fractions using 20% trichloroacetic acid, the samples were separated by ultracentrifugation. Proteins were precipitated from the cytosolic and membrane fractions using 20% trichloroacetic acid, the samples were separated into the nuclear, cytosolic, and membrane fractions. As mentioned before, Y16A and W208A showed a comparable, 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 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 nuclear 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...
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. Tyr72, Tyr120, Glu176, and Trp208 appear to be required only for the genomic DNA fragmentation activity. Tyr24 can be dispensed with completely, and Tyr160 and Glu176 partially, for RNA cleavage activity. The residue Arg24 can be dispensed with for both of the catalytic activities, namely RNA N-glycosidase and genomic DNA cleavage activity. Tyr24 does not seem to be playing any role in any of these activities of saporin-6. The crystal structure of saporin-6 and Arg179 are absolutely indispensable for RNA N-glycosidase activity. However, Trp208 could be dispensable for both DNA and rRNA. Trp208 appears to be indispensable for RNA N-glycosidase and genomic DNA fragmentation activity. Tyr72, Tyr120, Glu176, and Arg179 are important for different adenine-containing substrates (21). Therefore, it appears that saporin-6 binds to DNA and RNA 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. 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 conformational essential for RNA N-glycosidase activity.

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