From as early as 1925, it was known that Tobacco Mosaic Virus (TMV) suspensions, when mixed with the extracts of several plants, failed to transmit infection to other plants. Following purification and identification of the antiviral principles of the extracts of two plants *Phytolacca americana* and *Dianthin caryophyllus*, it was further observed that these proteins, pokeweed antiviral protein and dianthin, respectively, were responsible for the protein synthesis inhibitory activity that was observed also in these extracts. Subsequently, several other RIPs, both of types I and II were demonstrated to inhibit TMV infection of tobacco leaves, though with differing potencies (Barbieri *et al.*, 1993). However, although an antiviral activity has been established for several RIPs, the exact underlying mechanism is not known.
The saporin leaf isoform L1 was found to release adenines from several alternative substrates including TMV and bacteriophage MS2 genomic RNA, as well as globin mRNA and yeast tRNA and it was suggested that saporins could directly inhibit viral replication by damaging their genomic or messenger RNAs (Barbieri et al., 1994). Tricosanthin was the first type I RIP that was found to inhibit HIV-1 replication (McGrath et al., 1989) and subsequently several other findings of viral replication inhibition by different RIPs, such as pokeweed antiviral protein, GAP31 (Gelonium anti-HIV protein of 31 kDa), agrostin, luffin and saporin have been reported (Zarling et al., 1990, Lee-Huang et al., 1994, Wang et al., 2001). Studies with tricosanthin mutants and GAP31 peptide fragments, however, revealed that there was a disparity of sequence requirements for inhibition of HIV-1 infection and replication and ribosome-inactivating activity, suggesting that the anti-HIV activity of most ribosome-inactivating proteins may not be the result of N-glycosidase activity alone (Lee-Huang et al., 1994). The two RIPS, MAP30, Momordica anti-HIV protein of 30 kDa, and GAP31, Gelonium anti-HIV protein of 31kDa, also exerted a topological activity on plasmid and viral DNAs including viral long terminal repeats or LTRs (Lee-Huang et al., 1994). LTRs are essential sites for the integration of viral DNA into the host genome by the virus-encoded enzyme integrase. Both MAP30 and GAP31 were also found to exhibit a dose-dependent inhibition of HIV-1 integrase (Lee-Huang et al., 1995). Integration of viral DNA into the host chromosome is a vital step in the replicative cycle of retroviruses, including the AIDS virus, and the findings suggested that the impediment of viral DNA integration may play a key role in the anti-HIV activity of these plant proteins (Lee-Huang et al., 1995). Subsequently, two more plant RIPS, saporin and luffin were found to be the most potent inhibitors of
HIV-1 integrase among several RIPs investigated, and it was proposed that the differential HIV-1 integrase inhibitory activities of all these RIPs may be due to their different DNA glycosylase activity (Au et al., 2000).

Integration requires the virus-encoded integrase protein, as well as DNA sequences at the end of the linear viral DNA, located in the U3 and U5 regions of viral LTRs. A highly conserved feature of all retroviruses is the presence of a CA dinucleotide two bases from the 3' end of both U3 and U5 (Chow, 1997). The pathway of retroviral DNA integration reaction has been analyzed both in vivo and in vitro and proceeds in three steps. The first step is 3'-end processing, where two nucleotides are removed from the 3'-end of each strand of linear viral DNA, so that the viral 3' ends terminate with the conserved CA dinucleotide. The second step, 3'-end joining or strand transfer, is a concerted cleavage-ligation reaction during which integrase makes a staggered cut in the target DNA and ligates the recessed 3'-OH ends of the viral DNA to the overhanging 5'-P ends of the target DNA at the cleavage site. The product of the reaction is a gapped intermediate. The last step is 5'-end joining, where the integration process is completed by the removal of the two unpaired nucleotides at the 5'-ends of the viral DNA and repair of the gaps between the viral and target DNA. A schematic diagram of the pathway of retroviral DNA integration is depicted as Figure 5.1. The protein factors involved in the last step of the reaction are not yet characterized but are generally believed to be unspecified host repair enzymes (Chow, 1997). In vitro studies of purified integrases of several retroviruses, including HIV-1, and oligonucleotides containing DNA sequences of viral ends as substrates, have shown that purified integrase alone can catalyze the two
Figure 5.1
PATHWAY OF RETROVIRAL DNA INTEGRATION

Pathway of retroviral DNA integration. Thick lines represent viral DNA, and thin lines represent target DNA. The conserved CA dinucleotide at the viral DNA end is indicated. Filled triangles indicate sites of cleavage or 3'-end joining. Long vertical lines represent base pairs, and short vertical lines represent unpaired nucleotides.

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Characterization of saporin and mutants for antiviral activity

major steps of integration observed in vivo, namely, 3'-end processing and 3'-end joining (Bushman et al, 1991).

The present study was conducted to investigate the role of N-glycosidase activity of the RIP saporin in the HIV-1 integrase inhibition. The saporin seed isoforms 6 and 5, displaying differential catalytic activity but having conserved active site residues, the saporin-6 isoform mutant N162D, catalytically more similar to saporin-5, a few catalytically inactive active site mutants of saporin-6, and the fungal ribotoxin restrictocin, which recognizes the same target loop of rRNA as RIPS but is catalytically a ribonuclease and not an N-glycosidase, were investigated with regard to their anti-HIV integrase activity to ascertain whether this activity correlated with the classical catalytic activities of these proteins or not.

EXPERIMENTAL PROCEDURES

Purification of recombinant HIV-1 integrase

The plasmid pT7-7-His(TX)-HIV-1-IN, containing the DNA encoding HIV-1 integrase with a sequence coding for 6 histidine residues at its 5’ end, was used to express His-tagged HIV-1 integrase in BL21 Codon Plus (λDE3)-RIL strain of E. coli. Bacterial cells were transformed with the plasmid and grown in Luria-Bertini broth containing 50 µg/ml ampicillin and 50 µg/ml chloramphenicol at 25°C with shaking. The harvested cells were lysed by a combination of lysozyme treatment and sonication, and the pellet fraction obtained after centrifuging the lysate was homogenized in a high salt buffer, incubated at 4°C, and cleared by centrifugation. The supernatant containing integrase was loaded onto a 3 ml Ni-NTA (Qiagen) column and the bound protein was eluted with a 20 mM Tris-HCl, pH 8.0, 2M NaCl,
and 2mM β-mercaptoethanol buffer containing 200 mM imidazole. Protein containing fractions were pooled, EDTA was added to a final concentration of 5 mM, and the protein was dialyzed against a 20 mM HEPES, pH 7.5, 1 mM EDTA, and 1 M NaCl buffer, containing 20% glycerol and 2 mM β-mercaptoethanol, and then dialyzed against the same buffer containing 1 mM DTT instead of β-mercaptoethanol. The protein obtained after Ni-NTA column chromatography was partially pure (Figure 5.2) and the yield was 0.7 mg/l of culture.

**Preparation of dinucleotide substrate for HIV-1 integrase activity assay**

The 21-mer oligonucleotide HU5V1 (comprising sequences derived from the U5 end of HIV-1 LTR) was labeled at its 5'-end with $^{32}$P, in a typical phosphorylation reaction using [γ-$^{32}$P] ATP as the donor, in the presence of T4 polynucleotide kinase. After heat-inactivation of the kinase, the labeled oligonucleotide was purified from excess of [γ-$^{32}$P] ATP using a sephadex G-15 column. The purified oligonucleotide was then annealed to an equal amount of the complementary 21-mer oligonucleotide HU5V2, to form the double-stranded product, HU5V1:HU5V2. Aliquots of the annealed, radiolabelled product were stored at -20°C.

**RESULTS**

**Characterization of recombinant HIV-1 integrase for 3'-end processing and 3'-end joining activities**

The partially purified HIV-1 integrase was incubated with the labeled substrate for an appropriate time interval, and the reaction product, after heating in a formamide-containing loading buffer, was analyzed by electrophoresis using a 20%
The purified protein was analyzed on a 12.5% SDS-polyacrylamide gel stained with Coomassie blue. Molecular weight markers are indicated in kDa on the left. The arrow indicates the partially purified integrase which ran as an approximately 30 kDa band in the 12.5% gel.
polyacrylamide gel containing 7M urea. The gel was dried and autoradiographs of appropriate time intervals were developed. The 3'-end processing activity of integrase removed the last two nucleotides from the 3'-end of the 5'-end labeled substrate, generating a product that was shortened by two nucleotides (Figure 5.3 A). Furthermore, since the processed substrate was capable of mediating 3'-end joining using another substrate molecule as the target in the presence of integrase, 3'-end joining products of higher molecular weight as compared to the input substrate could also be visualized using the same gel, but with higher exposure times of the film (Figure 5.3 B).

**Characterization of saporin isoforms 6, 5 and isoform and active site mutants for integrase-inhibitory activity**

The two saporin isoforms 6 and 5, the saporin-6 isoform mutant N162D which showed a catalytic activity that was lower than that of saporin-6 and more comparable to saporin-5, as well as three catalytically inactive mutants of saporin-6, Y16A, Y72A and R179A were tested for their HIV-1 integrase inhibitory activity. The fungal ribotoxin restrictocin, ribonuclease A, as well as BSA were also tested as controls. 0.5 μg of integrase was incubated with 50, 100, 500 and 1000 ng of the various proteins for two hours at 37°C and reaction products analyzed on 20% polyacrylamide gel containing 7M urea as described in Materials and Methods. Autoradiographs were developed by exposing the film for 3h and 15h, to determine respectively the 3'-end processing and the 3'-end joining activities of integrase after treatment with various toxins. Despite the varying catalytic activities of the wild-type saporin isoforms and the different saporin mutants, they inhibited both 3'-end processing as well as 3'-end
3'-end processing activity of HIV-1 integrase was studied using the 21-mer dinucleotide substrate HU5V1:HU5V2, with the HU5V1 strand labelled at the 5' end with [γ-^32P]ATP. The conserved CA dinucleotide sequence is underlined. Activity is indicated by the appearance of a product that is shortened by two nucleotides, as seen in the one-hour autoradiograph. (A)

3'-end joining activity was studied using the same HU5V1:HU5V2 substrate. Activity is indicated by the appearance of products that are longer in length than the input DNA, as seen in the eight-hour autoradiograph. (B)

S=substrate, I=integrase, IP=integration products
joining activities of HIV-1 integrase with equal efficiency, with almost complete inhibition being observed when 500 and 1000 ng of protein was incubated with integrase (Figures 5.4, 5.5). The fungal ribotoxin restrictocin was also found to inhibit HIV-1 integrase activity to a similar extent as saporin-6 (Figure 5.6), while BSA and RNase A failed to elicit any inhibition of integrase (Figure 5.7).

**DISCUSSION**

Au et al, 2000, examined several common plant RIPs for their ability to interfere with HIV-1 replication by a series of mechanistic assays *in vitro* and observed that these RIPs inhibited integrase activity by 26-96%, with the most potent inhibitors being luffin and saporin, which caused more than 90% inhibition in an ELISA-based assay. Further characterization of these two RIPs by a radiometric assay showed a strong dose-dependent inhibition of HIV-1 integrase activity by these two RIPs (Au et al, 2000). The present study was conducted to investigate HIV-1 integrase inhibitory activity of saporin-6, with an aim to correlate it to its catalytic activity.

Y72A and R179A are active site mutants of saporin-6 which are catalytically inactive. In the inactive mutant Y16A, residue Tyr16, which lies outside the active site, but is needed to maintain a proper structural orientation of two active site residues in the solvent of the active site cleft (Bagga et al, 2003a), is replaced by alanine. The fungal ribotoxin restrictocin is a ribonuclease that cleaves a phosphodiester bond in the conserved target loop of 28S rRNA that is also attacked by the RIPs. The latter remove A4324 in this conserved loop by their N-glycosidase activity, while
Figure 5.4: EFFECT OF SAPORIN ISOFORMS AND MUTANT N162D ON HIV-1 INTEGRASE ACTIVITY

The dinucleotide substrate HU5V1:HU5V2 was incubated alone, with 0.5 μg of integrase, and with 0.5 μg of integrase in the presence of increasing amounts of the saporin isoforms and mutant as indicated. Samples were resolved on 20% polyacrylamide gel containing 7M urea and autoradiographs were developed after 3 hrs and 15 hrs of exposure of the film, to detect 3'-end processing and 3'-end joining activities respectively. S = substrate, I = integrase.
Figure 5.5: EFFECT OF CATALYTICALLY INACTIVE SAPORIN MUTANTS ON HIV-1 INTEGRASE ACTIVITY

<table>
<thead>
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<th>100</th>
<th>500</th>
<th>1000</th>
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<tbody>
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</tr>
<tr>
<td>S</td>
<td>+</td>
<td>+</td>
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<td>Y72A</td>
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<td>R179A</td>
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The dinucleotide substrate HU5V1:HU5V2 was incubated alone, with 0.5 μg of integrase, and with 0.5 μg of integrase in the presence of increasing amounts of the saporin mutants as indicated. Samples were resolved as before and autoradiographs were developed after 3 hrs and 15 hrs of exposure of the film, to detect 3'-end processing and 3'-end joining activities respectively. S = substrate, I = integrase.
The dinucleotide substrate HU5V1:HU5V2 was incubated alone, with 0.5 μg of integrase, and with 0.5 μg of integrase in the presence of increasing amounts of saporin and restrictocin as indicated. Samples were resolved as before and autoradiographs were developed after 3 hrs and 15 hrs of exposure of the film, to detect 3'-end processing and 3'-end joining activities respectively. S = substrate, I = integrase
The dinucleotide substrate HU5V1:HU5V2 was incubated alone, with 0.5 µg of integrase, and with 0.5 µg of integrase in the presence of increasing amounts of saporin, BSA and RNase A as indicated. Samples were resolved as before and autoradiographs were developed after 3 hrs and 15 hrs of exposure of the film, to detect 3'-end processing and 3'-end joining activities respectively. S = substrate, I = integrase
restrictocin cleaves the phosphodiester bond between G₄₃₂₅ and A₄₃₂₆ in the same highly conserved tetranucleotide GAGA sequence of this loop. All these above proteins, along with saporin isoforms 5, 6 and saporin-6 mutant N162D which was found to be catalytically similar to saporin-5 (Chapter One), were investigated in the present study. Irrespective of their varying catalytic RNA N-glycosidase activities, all these proteins could inhibit both the 3'‐end processing as well as the 3'‐end joining activities of HIV‐1 integrase with equal potency. Fungal ribotoxin restrictocin, a true ribonuclease, could also inhibit integrase activity as potently as saporin-6.

The first report of RIPs that inhibited HIV‐1 integrase activity were that of the proteins MAP30 and GAP31 (Lee-Huang et al, 1995) that were purified respectively from the plants Momordica charantia and Gelonium multiflorum. These two proteins exerted an overall antiviral effect by inhibiting HIV‐1 infection in T lymphocytes and monocytes as well as inhibiting replication of the virus in already infected cells (Lee-Huang et al, 2005). At least three separate activities of these two proteins were proposed to contribute to their antiviral activity (Lee-Huang et al, 2005); the first was the classical RNA N-glycosidase activity of these RIPs that could depurinate major rRNA. The second activity pertained to their ability to irreversibly relax and decatenate supercoiled DNA of both plasmids and viral LTR, as well as to their ability to cause double-stand breaks in DNA to form linear DNA (Huang et al, 1992). The relaxed DNA molecules generated by these proteins were topologically inactive and could no longer serve as substrates for DNA gyrase to form supercoils. It was proposed that the ability of these proteins to interrupt essential topological interconversions of DNA may provide a novel mechanism for their antiviral actions (Huang et al, 1992). The third activity was the ability of these proteins to inhibit the
3'-end processing, 3'-end joining or strand transfer, and disintegration activities of HIV-1 integrase (Lee-Huang et al, 1995). The findings of these workers indicated that these RIPs could block the specific actions of integrase in the U3 and U5 regions of HIV-1 LTR, that they were effective in interrupting the positioning of the 3'-end of the viral substrate to the target DNA independent of 3'-end processing, and that these proteins could block the release of the viral DNA from the integration intermediate, independent of the other two reactions. \textit{In vivo} studies have suggested that retroviral integration takes place preferentially at regions that are transcriptionally active, and also at DNase I hypersensitive sites (Kirchner et al, 1995). HIV-I integrase, MAP30 and GAP31 were all capable to binding to viral as well as target DNA substrates (Lee-Huang et al, 1995), and were equally capable of converting supercoiled DNA into relaxed and nicked forms. While integrase prepared the substrate DNA for viral integration, the RIPs could block the recombination site and prevent viral integration (Lee-Huang et al, 1995).

Although saporin has not yet been shown to exert any overall antiviral effect such as an inhibition of viral infection or replication, this molecule is an RNA N-glycosidase, exerts a non-specific DNase-like activity that can convert supercoiled DNA into nicked and linear forms, and could inhibit both 3'-end processing and strand transfer activities of integrase, similar to Map30 and GAP31. However, the RNA N-glycosidase activity of the protein does not seem to be a prerequisite for its activity in blocking integrase, as the mutants Y72A, R179A and Y16A, which were very weak N-glycosidases, were equally capable of inhibiting integrase. Saporin isoforms 6, 5, and several of the mutants tested displayed a DNase-like activity, though to varying extents. It is therefore possible that a DNase-like activity is more crucial for integrase
inhibition. Restrictocin, a strong and specific ribonuclease, could inhibit integrase, while RNase A, a strong, non-specific ribonuclease, could not. The mechanism of integrase inhibition by restrictocin should be explored by checking whether this protein also has a nuclease-like activity.