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Interactions between saporin, a ribosome-inactivating protein, and DNA: a study by atomic force microscopy

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Summary

Saporins are enzymes belonging to the PNAG class (polynucleotide: adenosine glycosidase), plant enzymes commonly known as ribosome-inactivating proteins (RIP), as a result of their property of irreversibly damaging eukaryotic ribosomes. Direct imaging with tapping-mode atomic force microscopy (AFM) has been used to study pGEM-4Z plasmid DNA binding to the saporin-SO6 (isoform from *Saponaria officinalis* seeds). Saporin wrapped the plasmidic DNA, and distribution of the enzyme molecules along the DNA chain was markedly variable; plasmid digested with saporin-SO6 appeared fragmented or topologically modified. The supercoiled DNA strands were cleaved, giving rise to a linearized form and to relaxed forms. Electrophoretic analysis of the effect of standard preparations of saporin-SO6 on pGEM-4S confirmed the presence of DNA strand-cleaving activity.

Introduction

Saporins are enzymes belonging to the PNAG class (polynucleotide: adenosine glycosidase) (Peumans *et al.*, 2001), plant enzymes commonly known as ribosome-inactivating proteins (RIP) (Barbieri *et al.*, 1993), owing to their property of irreversibly damaging eukaryotic ribosomes. RIPs are classified into three groups (Nielsen & Boston, 2001); type I consists of a single chain with a molecular weight of 25 000–30 000 Da; type II, with a molecular weight of 60 000 Da, usually consists of two chains (A and B) connected by a disulphide bond – the B chain binds to galactose-containing receptors on cell surfaces and facilitates the entry of the A chain into the cytoplasm, where it inactivates the ribosome; type III are single-chain proteins synthetized as inactive zymogenes that require proteolytic processing of internal regions to form active proteins (Walsh *et al.*, 1991; Mundy *et al.*, 1994). PNAG enzymes have been previously identified as rRNA *N*-glycosidases in the enzyme classification (EC 3.2.2.22) because the larger rRNA molecule in intact eukaryotic ribosomes was the first substrate to be identified (Reisbig *et al.*, 1981). These enzymes remove a single adenine (A4324) from 28S rRNA in rat ribosomes, or its equivalent in other species, located at a stem and loop region highly conserved throughout evolution. Since the early studies on their mechanism of action in whole cells, the existence of substrate(s) other than ribosomes was suspected, and evidence for this has increasingly accumulated (Barbieri *et al.*, 1997).

These typical plant proteins have received a great deal of attention in biological and biomedical research because of their unique biological activities toward animal and human cells (Poma *et al.*, 1999a,b; 2001). In addition, evidence is accumulating that some RIPs play a role in plant defence and hence can be exploited in plant protection (Nielsen & Boston, 2001).

DNA has been proposed as an additional target of PNAG, although its importance and the mechanisms by which PNAG act on DNA are still unresolved. Determination of PNAG activity on DNA is relevant to studies of the mechanism of action at the molecular level, to studies of anti-viral (including anti-HIV) activity and to studies of the induction of apoptosis by PNAG. Several RIPs were reported to cleave and to linearize *in vitro* single-stranded M13 phage DNA (Roncuzzi & Gasperi-Campani, 1996) as well as supercoiled DNA (Ling *et al.*, 1994). In general, the observed DNase activity occurs only at high RIP concentrations. It is also believed that in contrast to RNA *N*-glycosidase activity, recognition of the supercoiled or single-stranded DNA as a substrate for nuclease activity is not site specific, but may be determined by the three-dimensional structure of the DNA.

The atomic force microscope (AFM) is the most versatile member of the family of scanning probe microscopes (SPMs). SPMs are not traditional microscopes: they visualize surfaces by scanning them with a sharp probe; conventional microscopes image by collecting radiation transmitted through, or reflected

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from, the sample. SPM images, by contrast, are obtained by measuring changes in the magnitude of the interaction between the probe and the specimen surface as the surface is scanned with the probe. With the AFM there are fewer restrictions on specimen size, and it is therefore possible to examine biological samples (not necessarily conducting samples) ranging in size from individual molecules to cells or tissue in their native state. DNA is one of the most studied biopolymers using SPM methods; in order to gain the full advantages of the AFM, it would be preferable to investigate these molecules under natural conditions, usually in aqueous or buffered environments. Because of its fundamental role in biology, DNA has been studied extensively by AFM (Hansma *et al.*, 1995). The most common substrate for AFM imaging of DNA is mica; this consists of a series of thin flat crystalline plates that can easily be split apart ('cleaved') by inserting a pin at its edge. Mica sheets are atomically flat over large areas (several micrometres), which is essential if molecular resolution is to be achieved. In this study direct imaging with the AFM has been used to characterize saporin/pGEM-4S plasmid binding.

In parallel, electrophoretic methods are given to confirm saporin activity on plasmid DNA.

Materials and methods

Saporin purification

Standard saporin preparations from *Saponaria officinalis* seeds were obtained following the method described in Poma *et al.* (1999b). The saporin inhibitory effect on *in vitro* translation was determined by measuring the incorporation of L-[^{3}H]leucine into proteins using a rabbit reticulocyte lysate cell-free system (Promega) (Poma *et al.*, 1999b). The reaction was carried out for 30 min at 37 °C, in a final volume of 62.5 μL, followed by 20% trichloroacetic acid (TCA) precipitation. The saporin IC_{50} (50% inhibiting concentration) was 3 ng mL^{-1}.

AFM analysis

For protein observation, 1 μL saporin-SO6, 0.01 μg μL^{-1} resuspended in phosphate buffer, was deposited onto a 1.5-cm^2 disc of freshly cleaved ruby mica, dried in a normal dessicator with silica gel for 2 h and observed.

DNA, with its negatively charged phosphate backbone, was bound to the negatively charged mica surface by the addition of bivalent cations, which function as an electrostatic bridge. In addition to this effect, ions in the buffer will shield electrostatic charges and thus lower the repulsive force between DNA and mica. A compromise between firm and loose imobilization was achieved by optimizing the concentration and type of mono and bivalent cations. DNA (immobilized on mica in a buffer containing 1.5 mm $MgCl_2$, 50 mm KCl) was only partially bound to the mica surface at specific points; the loose parts moved over the surface and were free to interact with proteins.

For AFM analysis of pGEM-4Z (Promega) without saporin treatment we used a 5-μL solution of pGEM (10 ng μL^{-1}) in 10 μL buffer solution (10 mm Tris/HCl, pH 7.4, 1.5 mm $MgCl_2$, 50 mm KCl); 1-μL samples were deposited onto a 1.5-cm^2 disc of freshly cleaved ruby mica and left to adsorb for 1 min. The disc was then rinsed with a drop of 0.2% uranyl acetate and dried in a normal dessicator with silica gel for 2 h.

For the AFM analysis of pGEM-4Z depurinated by PNAG we used a 2-μL solution of saporin-SO6 (0.01 μg μL^{-1} resuspended in phosphate buffer) and a 5-μL solution of pGEM-4Z (10 ng μL^{-1}) in 10 μL buffer solution (10 mm Tris/HCl, pH 7.4, 1.5 mm $MgCl_2$, 50 mm KCl). The mixture was incubated at 37 °C for 60 min and the incubation was stopped by placing the sample microtube on ice for 2 min. The sample (1 μL) was deposited onto a 1.5-cm^2 disc of freshly cleaved ruby mica and left to adsorb for 1 min.

The disc was then rinsed with a drop of MilliQ filtered water and dried in an dessicator for 2 h.

Tapping-mode AFM images were collected on a NanoScope IIIa system equipped with a multimode head and a type-E piezoelectric scanner (Digital Instruments, Santa Barbara, CA, U.S.A.) using silicon probes for non-contact/tapping-mode applications.

Gel electrophoresis

The interaction between supercoiled pGEM-4Z DNA and saporin was analysed on 1% agarose gels. One microgram of pGEM-4Z was incubated with saporin (1.5 μg) in 10 μL Hepes buffer 10 mm and $MgCl_2$ 0.05 mm, pH 7.0, for 60 min at 37 °C. The samples were loaded onto 1% agarose gels, electrophoresed for 1 h (70 mA) and visualized by staining with ethidium bromide (1 μg mL^{-1}).

Results and discussion

AFM images of pGEM-4Z DNA (Fig. 1A–D) and of saporin-SO6 (Fig. 2) are shown. The images are stable under repeated scans, indicating that plasmid is strongly attached to the mica surface (Fig. 1A–D). In their native state, plasmids are supercoiled; supercoiling is an important biological phenomenon that is relevant for a number of processes (Bates & Maxwell, 1997). Originating from a topological constriction (generally underwinding of the two DNA chains), supercoiling translates into geometrical effects; twisting and writhing of the chains in three-dimensional space are altered. The free supercoiled DNA in solution is expected to have an interwound shape termed 'plectonemic' that can be explained by considering the DNA as an elastic chain. The shape of supercoiled plasmids as revealed by AFM images is often very open, with only one or two apparent crossovers; it is sometimes difficult to distinguish the loosely coiled plasmids from relaxed plasmids that can have 'random flops' especially if they are very long. In the conditions of DNA deposition and dehydration adopted for AFM imaging,

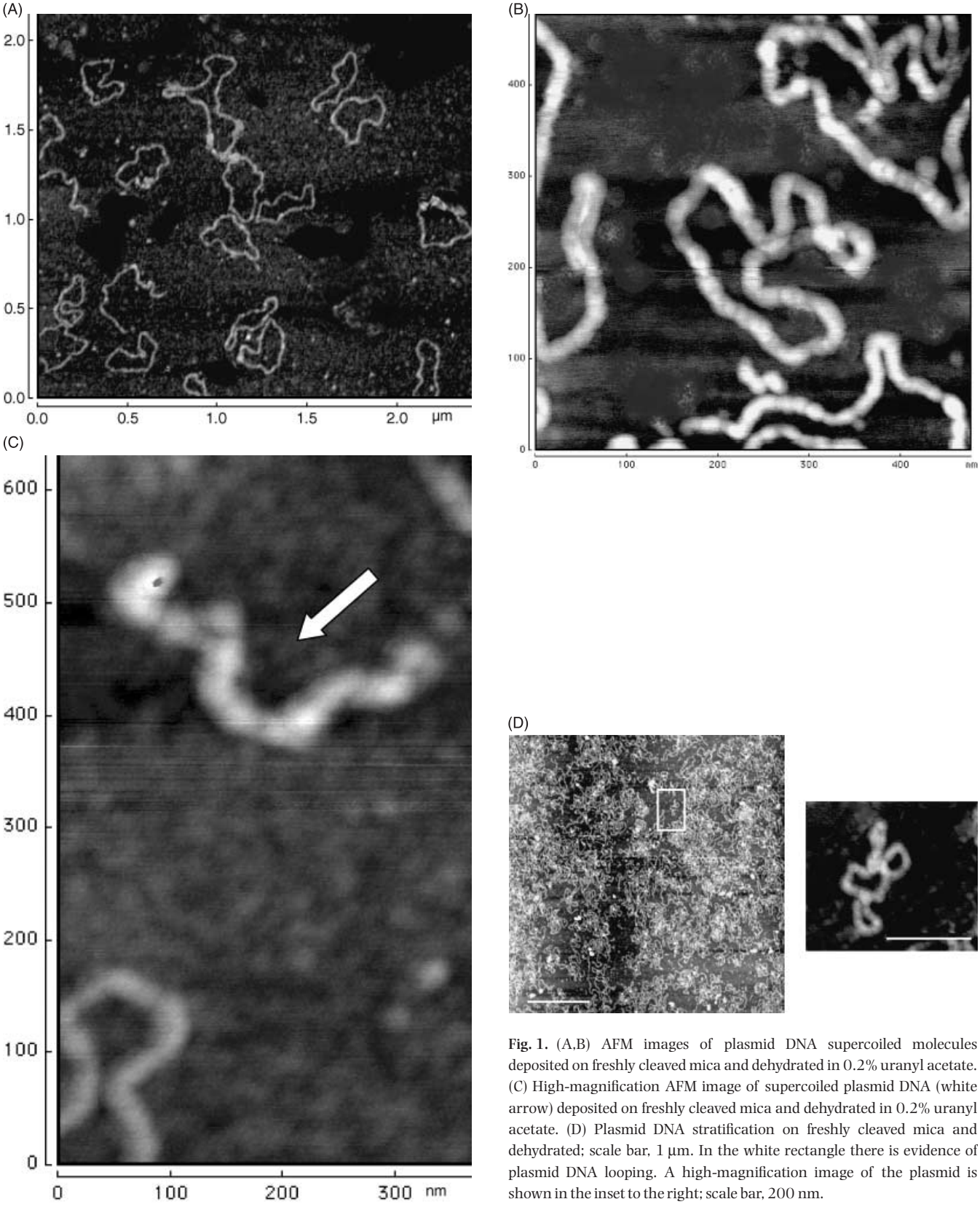

Fig. 1. (A,B) AFM images of plasmid DNA supercoiled molecules deposited on freshly cleaved mica and dehydrated in 0.2% uranyl acetate. (C) High-magnification AFM image of supercoiled plasmid DNA (white arrow) deposited on freshly cleaved mica and dehydrated in 0.2% uranyl acetate. (D) Plasmid DNA stratification on freshly cleaved mica and dehydrated; scale bar, 1 µm. In the white rectangle there is evidence of plasmid DNA looping. A high-magnification image of the plasmid is shown in the inset to the right; scale bar, 200 nm.

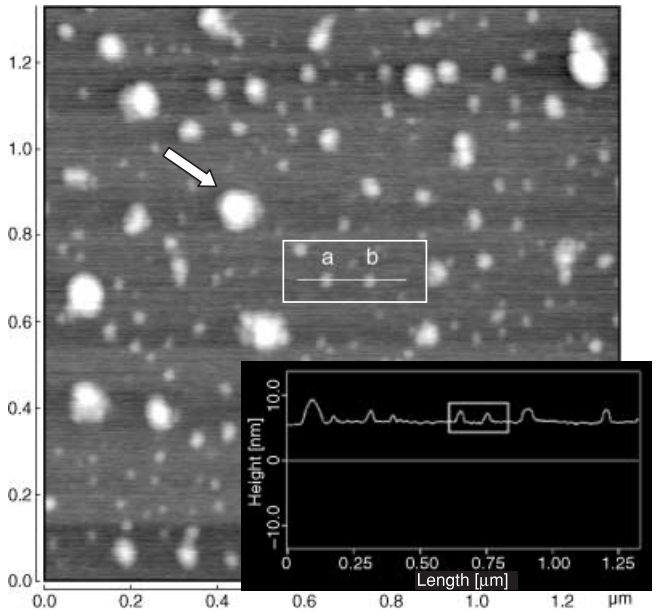

Fig. 2. AFM image of saporin stratification with the vertical profile of two different molecules of the protein (a and b).

some condensation may occur, especially if this is aided by the superhelical tension. These conditions can even drive the molecules towards unnatural shapes (Fig. 1C,D). Evidence is accumulating that in the normal conditions of imaging the observed shapes are usually fairly loose. If the ionic strength of the solution used to deposit the DNA is very high, then the deposited plasmids can appear as highly coiled (Lyubchenko *et al.*, 1997).

Plasmid treatment with uranyl acetate is used by us in the dehydratation phase to preserve the structure of the molecules; in this case the plasmids can be highly coiled, and it is very easy to distinguish between relaxed and supercoiled plasmids. Statistical evaluation of the two populations is thus very similar to quantitative analysis by agarose gel electrophoresis.

At high ionic strength conditions, plasmids are extremely coiled; they can been seen as coiled on the surfaces, especially if the experimental conditions (AP-mica or uranyl acetate) limit their mobility before the ionic conditions are altered. Uranyl acetate might have the dual role of limiting the mobility and increasing the ionic strength.

An AFM image of saporin stratification with the vertical profile of two different molecules of the protein (a and b) is shown in

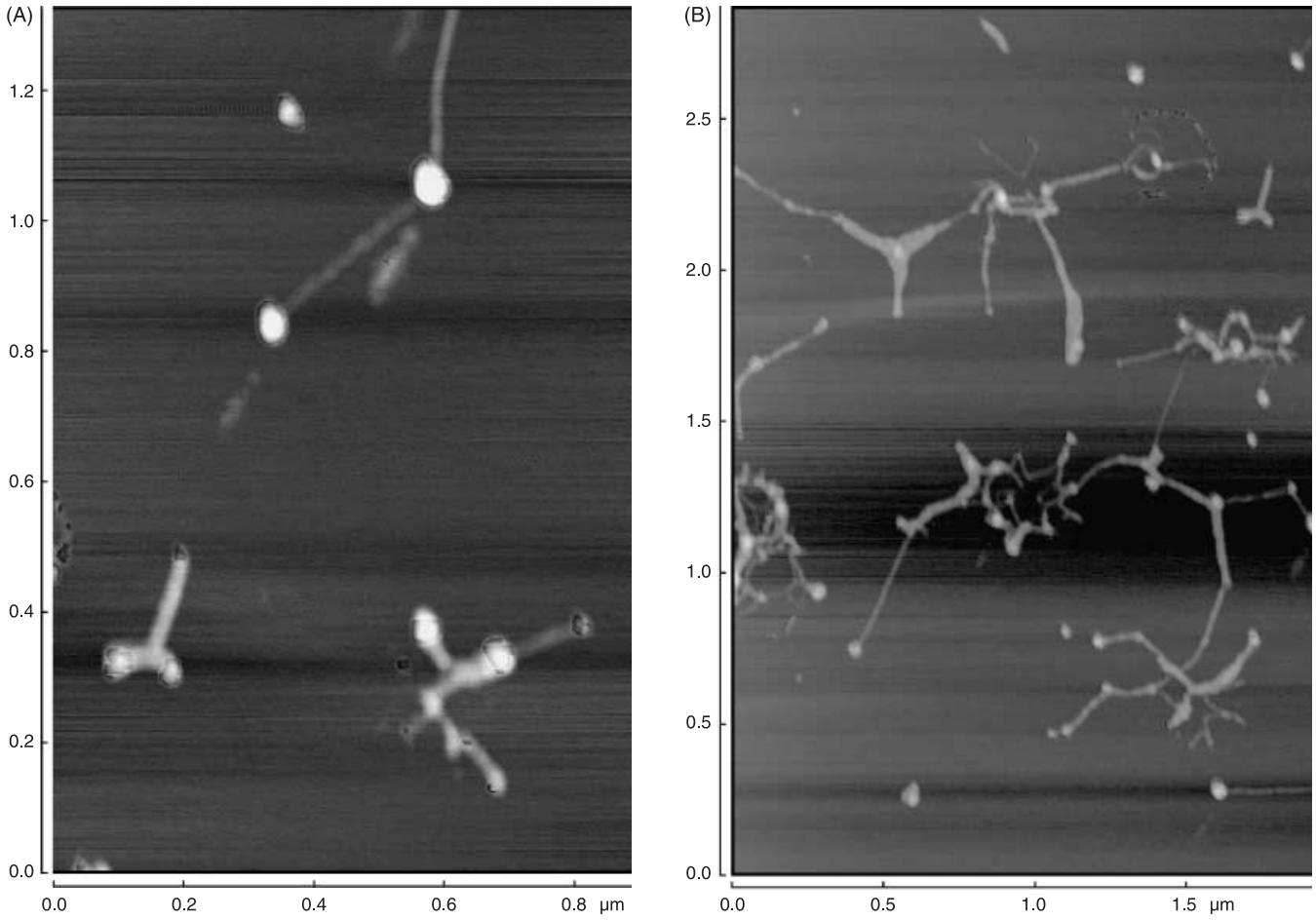

Fig. 3. (A,B) AFM images of nicked pGEM-4Z molecules after 60 min of incubation with saporin-SO6.

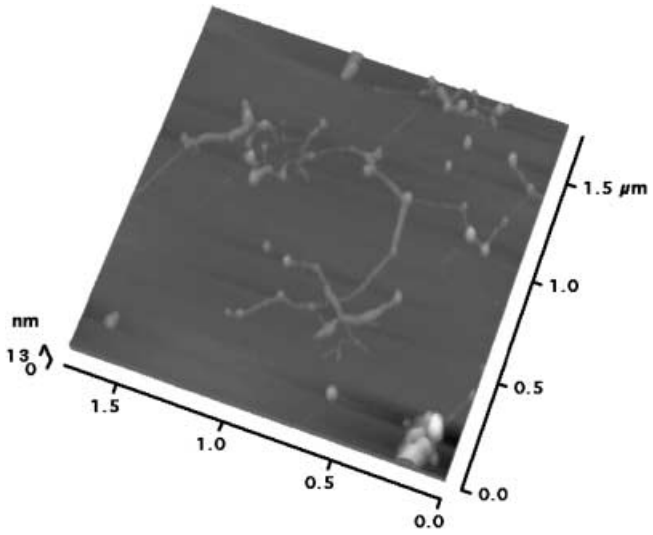

Fig. 4. Three-dimensional representation of nicked pGEM-4Z molecules after 60 min of incubation with saporin-SO6.

Fig. 2; it is possible to see saporin aggregates (white arrow) resulting from adhesive forces between proteins and the mica surface.

Many protein molecules are bound to the same plasmid fragment, and the distribution of the bound saporin-SO6 molecules along the linearized plasmid is markedly variable (Fig. 3A,B); plasmids digested with saporin-SO6 appear fragmented or topologically modified. Figure 4 shows a 3D image of nicked pGEM-4Z molecules after 60 min of incubation with saporin-SO6. The enzyme binds extensively to the plasmidic DNA. AFM analysis of pGEM after saporin treatment gives morphological confirmation that saporin activity causes cleavage of double strands. A histogram of the diameter of stratified saporin molecules is given in Fig. 5(A) (mean 40 ± 20 nm), and of saporin aggregates after 1 h of incubation with pGEM is shown in Fig. 5(B) (mean 70 ± 20 nm). The sizes of different saporin molecules, free (Fig. 5A) and bound to the plasmid (Fig. 5B), could be explained by protein interaction, with the double DNA strand producing twice the diameter of the bound molecules.

Electrophoretic analysis of the effect of standard preparations of saporin SO6 on pGEM-4S confirmed the presence of DNA strand-cleaving activity (Fig. 6). The supercoiled DNA strands (Figs 6, S bands) were cleaved, giving rise to a linearized form (Fig. 6, L bands) and to relaxed forms (Fig. 6, R bands). Some authors suggest that the DNase activity of RIPs is due to the presence of contaminating nucleases (e.g. Day *et al.*, 1998). Our results demonstrate first that saporin is able to cleave supercoiled DNA and second that its ability is not due to the presence of contaminating nucleases, because in addition the recombinant form of the wild-type enzyme is active on the same substrate; DNase activity assays of native and recombinant saporin indicate that both cleave supercoiled double-stranded

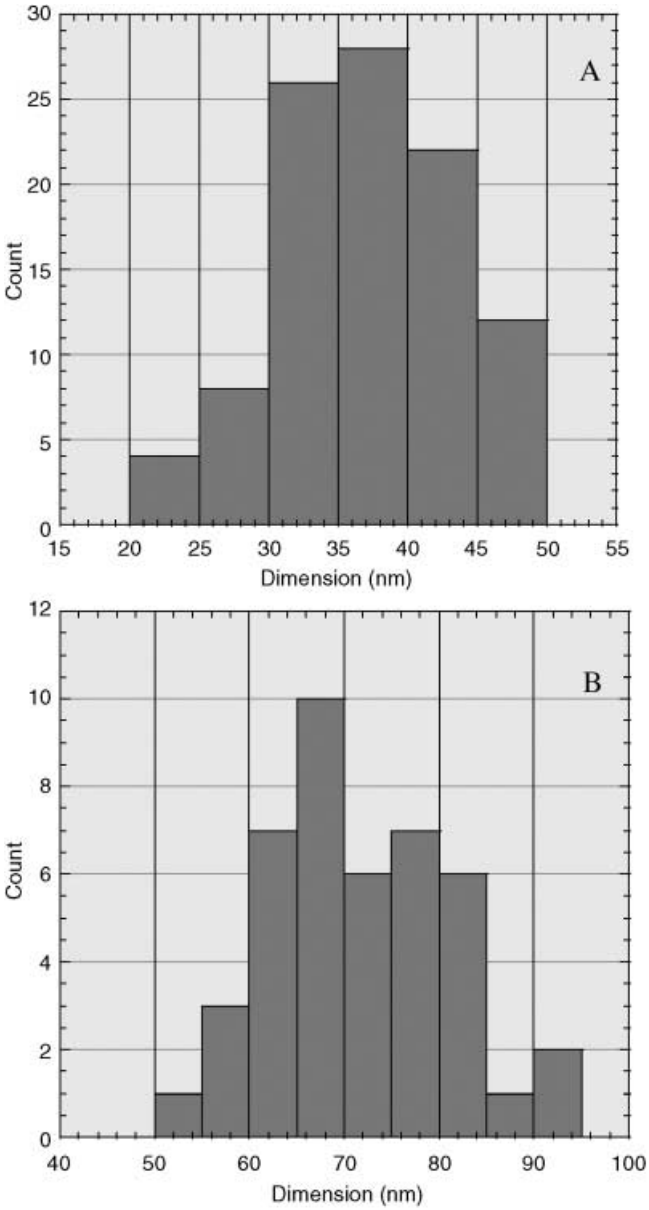

Fig. 5. Diameters of single saporin molecules (A) and saporin aggregates after 1 h of incubation with pGEM (B).

DNA into relaxed and linear forms *in vitro* (our unpublished data). Our observations by AFM show for the first time that saporin molecules interact with pGEM-4Z DNA. Another RIP, nigrin b, promoted the conversion of supercoiled pGEM-4Z DNA into both relaxed-circle and linear DNA. This indicated that basic nigrin b also displays the topoisomerase activity found in some RIPs, which seems to be involved in a significant way in their anti-HIV-1 action (De Benito *et al.*, 1997). These data are consistent also with the fact that the pokeweed antiviral protein exhibits an enzymatic activity on cleaving supercoiled double-stranded DNA into the nicked or linear form using the same active site that is required to depurinate rRNA (Wang &

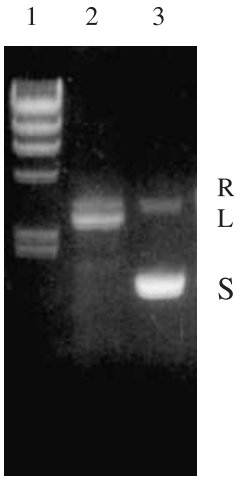

Fig. 6. Agarose gel electrophoresis of pGEM-4Z DNA treated with saporin. The samples were run on 1% agarose gels and visualized by staining with ethidium bromide. Lane 1: molecular mass marker. Lane 2: pGEM-4Z treated with saporin; the upper band is nicked relaxed plasmid (R) and the middle band linear plasmid (L). Lane 3: undigested pGEM-4Z; the fastest migrating band corresponds to supercoiled plasmid (S).

Tumer, 1999). Our findings demonstrate that saporin is able to bind supercoiled DNA, to nick these regions and to transform supercoiled into relaxed and linear molecules. We conclude that SO6 acts as a supercoil-dependent endonuclease when it cleaves supercoiled pGEM-4Z DNA.

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