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N-Terminal Deletion Affects Catalytic Activity of Saporin Toxin

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Abstract Single-chain ribosome inactivating proteins (RIPs) are cytotoxic components of macromolecular pharmaceutics for immunotherapy of cancer and other human diseases. Saporin belongs to a family of single-chain RIPs sharing sequence and structure homology. In a preliminary attempt to define an active saporin polypeptide of minimum size we have generated proteins with deletions at the N-terminus and at the C-terminus. An N-terminal (sapΔ1–20) deletion mutant of saporin displayed defective catalytic activity, drastically reduced cytotoxicity but increased ability to interact with liposomes inducing their permeabilization at low pH. A C-terminal (sapΔ239–253) deletion mutant showed instead a moderate reduction in cytotoxic activity. A substantial alteration of secondary structure was evidenced by Fourier transformed infrared spectroscopy (FTIR) in the sapΔ1–20 mutant. It can be hypothesized that the defective functions of sapΔ1–20 are due to alterations of its spatial configuration. J. Cell. Biochem. 98: 1130–1139, 2006.
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Key words: saporin; catalytic activity; secondary structure; ribosome inactivating proteins

Ribosome inactivating proteins (RIPs) from plants are toxic translation inhibitors that inactivate ribosomes by catalyzing the hydrolysis of a specific N-glycosidic bond of large rRNA [Barbieri et al., 1993]. RIPs have been classified into two types; type I RIPs, for example, saporin, trichosanthin, and pokeweed antiviral protein (PAP), are single-chain proteins lacking a second cell-binding domain unlike their type II counterparts that include ricin and abrin. Because of the absence of a binding domain, the single-chain type I RIPs lack the non-specific cytotoxicity shown by the type II RIPs and are, therefore, toxins of choice for the construction of immunotoxins. Thus, they are useful for treating cancer and autoimmune diseases and also against HIV infection [McGrath et al., 1989]. By virtue of their antiviral properties, type I RIPs might also be used to improve defence mechanisms of transgenic plants of interest [Lodge et al., 1993; Taylor et al., 1994].

Structure comparisons between saporin and other RIPs revealed that the catalytic site and the general 3-D organization are very similar [Savino et al., 2000]. Several isoforms of saporin with different chromatographic properties have been described [Barbieri et al., 1993] and some of them have been obtained in recombinant form [Barthelemy et al., 1993]. The isoform SO6 conjugated with vehicle molecules has been successfully applied to eradicate target cells in vitro and in vivo [Barthelemy et al., 1993]. SO6 is made up of two domains, the N-terminal domain which is predominantly β-stranded, and the C-terminal domain which is predominantly α-helical. The N-terminal domain is very similar to that of other RIPs [Savino et al., 2000]. The

Abbreviations used: RIPs, ribosome inactivating proteins; SO6, major isoform of saporin extracted from seeds; RTA, ricin toxin A-chain; APRT, adenine phosphoribosyl transferase; LUVs, large unilamellar vesicles; FTIR, Fourier transformed infrared spectroscopy; PC, phosphatidylcoline; PA, phosphatidic acid.

Francesca Bonini and Roberta Traini contributed equally to this work.

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C-terminal domain contains eight α-helices with canonical geometry. The active site residues Glu176, Arg179, and Trp208 of saporin are completely superimposable with those of other RIPs and in the C-terminal region three lysyl residues (220, 226, and 234) have been identified which map a structural motif highly conserved in all RIPs and possibly involved in the enzyme-substrate molecular recognition mechanism [Savino et al., 2000]. Structure-based alignments show that positively charged or polar residues are located in the corresponding positions of the RIPs PAP, momordin (MOM), and ricin toxin A-chain (RTA). RTA is the most studied representative of a number of RIPs that are under investigation for application in human diseases. Thus, substantial efforts have been made to relate the structure of RTA to its mechanism of action. Using deletion mutants it was initially demonstrated that in RTA the sum of all aminoacids that can be removed from various constructs without loss of activity amounts to 83%, indicating that a large number of the residues is neither absolutely essential for folding into an effective conformation nor for catalysis [Morris and Wool, 1992]. However, deleting the first nine residues from the N-terminus of RTA did not affect toxicity whereas deleting further three residues inactivated the polypeptide [May et al., 1989]. Bagga et al. [2003a] have demonstrated that point mutation of Tyr 16 of saporin drastically affects its catalytic activity. Mutated saporin retained, however, a substantial cytotoxicity against cell cultures, indicating that other functions besides its N-glycosydase activity could be responsible for saporin effects or that other N-terminal residues may be involved in saporin functions and/or its structural stabilization. These studies reinforce the conviction that deletion analysis is a valuable procedure for defining the overall role of discrete protein domains in saporin function and the minimal cohort of aminoacids necessary for substrate recognition and for catalysis. Considering the structural similarities between RTA and sapoerin, we have set out to initiate a systematic study of structure-function relationships in saporin based on a deletion approach. In the present study, we have investigated the role of discrete saporin domains using one N-terminal and one C-terminal deletion mutants. The results obtained support the notion that the N-terminal 20 aminoacids are crucial for enzymatic activity and for folding.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Saporin Isoforms

Plasmid isolation, preparation of competent cells and transformation, and DNA manipulations were carried out according to published procedures [Sambrook et al., 1989].

To obtain saporin deletion mutants we used the genomic clone 4 (G4) [Barthelemy et al., 1993] cloned in pET-11d (Novagen). The construct used was pET-11d-SAP3 in which a *SacII-EcoRI* fragment from sequence three DNA [Barthelemy et al., 1993] was ligated to pET-11d-DNA digested with *EcoRI* and *NcoI* in the presence of a linker-adapter containing *NcoI-SacII* sites. The construct pET-11d-SAP3 [Fabbrini et al., 1997] was kindly supplied by Dr. S. Fabbrini (Department of Biological and Technological Research—Dibit, San Raffaele Scientific Institute, Milan, Italy). pET-11d-SAP3 was PCR-mutated to create two different mutants deleted of 20 aminoacids at the N-terminus (sapΔ1–20), and of 15 aminoacids at the C-terminus (sapΔ239–253), respectively.

Synthetic oligonucleotides. The primers used to obtain the mutant sapΔ1–20 were: (1) *Oligo*-"*sense*" 5′-GAGATATACCATGGTCACA-TCAATC-3′ (in which an *adapter* CCATGG corresponding to an *NcoI* restriction site was inserted for ease of cloning), (2) *oligo*-"*anti-sense*" CCGGAATTCTACACCTGCCTCAC-TT-TTCC (containing an *EcoRI* restriction site). The primers used to obtain the mutant sapΔ239–253 were: (1) *Oligo*-"*sense*" GCGCC-ATGGATAAAATCCGAAAC and (2) *oligo*-"*anti-sense*" TCTTGAAGAATTCGCCTC-GTT (which contains an *adapter* introducing the stop codon (TAG) and an *EcoRI* restriction site).

Polymerase chain reaction. Reactions were carried out with 0.5 μg of pET-11d-SAP3 in 50 μl, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, and 0.15 mM of each primer. One unit of DNA Polymerase AmpliTaqGold™ (Invitrogen, Carlsbad, CA) was then added and incubations carried out in a GeneAmp PCR System 2400 (Perkin-Elmer, Wellesley, MA). Amplification cycles consisted of a denaturation step (94°C for 1 min), an annealing step (55°C for 1 min), and an elongation step (72°C for 1 min). After 25 cycles an aliquot of each reaction was loaded onto a 0.8% agarose gel.

Transformation. The *Escherichia coli* strain BL21(DE3)pLysS (Novagen, Milan, Italy) was transformed with the different constructs of saporin according to the manufacturer's instructions.

Expression of saporin isoforms. Hundred milliliter of LB-medium, complemented with 100 μg/ml ampicillin and 35 μg/ml chloramphenicol, was inoculated with one transformed colony of *E. coli* strain BL21(DE3)pLysS and incubated overnight at 37°C, with stirring. Total volume was then brought to 1 L with LB-medium additioned with 100 μg/ml ampicillin and 50 μg/ml chloramphenicol, and the cells were incubated at 37°C. Expression of heterologous proteins was induced by adding 1 mM isopropyl-β-thiogalactopyranoside (IPTG) and subsequent incubation for 3 h at 30°C. Bacteria were then pelleted (5000 rpm, 15 min at 4°C) and lysed with 50 mM Tris, 2 mM EDTA, pH 8.0, complemented with 1× complete protease inhibitor (Roche, Basel, Switzerland). After lysis the cells were sonicated on ice in 3 × 45 s bursts using a Labsonic-U ultrasonic disintegrator (B. Braun Biotech International, Melsungen, Germany). Lysates were then centrifuged (20 min, 22,000 rpm at 4°C) and the supernatant dialyzed overnight against 5 mM Na_2HPO_4, 5 mM NaH_2PO_4 buffer pH 6.5 at 4°C.

Purification. The supernatant was filtered with a 0.5 μm Millipore filter to eliminate debris and subjected to FPLC chromatography with a High-S cartridge column (Bio-Rad, Richmond, CA) equilibrated in 5 mM phosphate buffer, pH 6.5, wild type (wt) saporin or its mutants were eluted with a 0–300 mM NaCl gradient. Saporin isoforms were then further purified by gel filtration chromatography with a Superdex 75 (Hi Load 16/60, Pharmacia, Uppsala, Sweden) column equilibrated in PBS and run at 1 ml/min.

Western blotting. The purity of the purified proteins was then checked by SDS–PAGE and Western blotting using an antisaporin antibody from rabbit (kindly supplied by Dr. Fabbrini). Analysis by SDS–PAGE was as described in [Sambrook et al., 1989] using 12.5% (w/v) acrylamide separating gels. For Western blot analysis, proteins transferred onto nitrocellulose were probed with a rabbit antirabbit-horseradish peroxiolase-conjugate antiserum (Sigma, St. Louis, MO). The major isoform of saporin isolated from seeds (SO6) was used in our investigation [Barbieri et al., 1993]. SO6 was kindly supplied by Dr. S. Fabbrini.

Determination of Enzymatic Activity

The enzymatic activity of saporin isoforms was determined as described in [Heisler et al., 2002] by measuring adenine release from a substrate in a colorimetric assay. Briefly, samples of saporin isoforms were separately incubated with denatured herring sperm DNA (Gibco BRL, Gaithersburg, MD) at different concentrations (0.5–50 pmol) for 2 h at 30°C. Ethanol (100 μl, −20°C) was then added and the precipitate was pelleted by centrifugation (13,000 rpm for 30′ at 4°C). The supernatant was vacuum-dried, resuspended in 50 μl water and transferred to modular 96-well microplates (Nunc, Wiesbaden, Germany). To each sample 100 μl of cold "basic master mix" (8 μl adenine phosphoribosyl transferase (APRT) (0.3 μg/μl), 2 μl purine nucleoside phosphorylase (PNP) (100 U/ml) (Molecular Probes, Eugene, OR), 3 μl 5-phospho-D-ribosyl-1α-pyrophosphate (PRPP) (10 mM) (Sigma), 50 μl 2-amino-6-mercapto-7-methylpurine ribonucleoside (MESG) (1 mM) (Molecular Probes), 17.5 μl reaction buffer (400 mM Tris, 20 mM magnesium chloride, 2 mM sodium azide) and 50 μl of cold "starter mix" (10 μl inorganic phosphatase (1 U/ml) (Sigma), 5 μl 5′-nucleotidase (5 U/ml) (Sigma), 2.5 μl reaction buffer (see above) were added to start inorganic phosphate release and thus the colorimetric reaction. The kinetics of adenine release was monitored every 5 min with a microplate reader (VERSA max, Molecular Device, Sunnyvale, CA). The adsorbance measured at 360 nm was converted into a turnover rate by using a standard curve of adenine concentration.

Cytotoxicity

The cytotoxic activity of saporin isoforms was compared in protein-synthesis-inhibition assays. Cells (Jurkat, human leukaemia, and DU145, human prostate carcinoma) were from the American Tissue Culture Collection (Rockville, MD). Protein synthesis was measured by dispensing 5×10^4 cells in leucine-free RPMI-1640 medium (complemented with FBS 10% and glutamine 5%) in 96-well flat-bottomed microtitration plates. Saporin isoforms (tenfold dilutions of toxins in 10 μl of PBS/BSA 0.2%) were then added (final volume, 100 μl) and samples tested in triplicates. Microcultures were incubated for 24 h, followed by a 4 h pulse with 1 μCi of [^{14}C]-leucine. At the end of the

assay, the cells were washed with water and harvested onto glass-fiber filters (Dynatech, Chantilly, VA). Radioactivity incorporated by the cells was then measured in a beta-spectrometer (Wallac 2406). Mock-treated controls supplied the incorporation values corresponding to 100% protein synthesis.

Interaction With Large Unilamellar Vesicles

The interaction of saporin and saporin mutants with model membranes was investigated using a membrane permeabilization assay of large unilamellar vesicles (LUVs) prepared by extrusion technique [MacDonald et al., 1991].

Preparation of vesicles. LUVs were made by phosphatidylcoline (PC), phosphatidic acid (PA) (Avanti Polar Lipids, Alabaster, AL) or PA and PC at a 1:1 molar ratio. Lipid films were formed by removing chloroform from a lipid solution in a rounded flask with rotary evaporation and final vacuum drying (1 h). Multilamellar liposomes (at a total lipid concentration of 5 mg/ml) were suspended in a solution containing 80 mM calcein and subjected to six cycles of freezing and thawing. Using a two-syringe extruder (liposofast Basic unit; Avestin, Ottawa, Canada) they were repeatedly (31 passages) passed through two-stacked polycarbonate filters bearing holes of 100-nm diameter (Millipore, Billerica, MA). To remove untrapped calcein, the vesicles were spun through minicolumns (Phenomenex, Torrance, CA) loaded with Sephadex G-50 (medium, Sigma) pre-equilibrated with 140 mM NaCl, 20 mM HEPES and 1 mM EDTA, pH 7.0. Dimension and homogeneity of the vesicles were routinely estimated by dynamic light scattering using a Malvern Zeta-Sizer three apparatus (Malvern Instruments, Malvern, UK).

Permeabilization assay. Permeabilization was determined by measuring the fluorescence of calcein released from the vesicles [Kayalar and Duzgunes, 1986], using a fluorescence microplate reader (Fluostar Galaxy, Offenburg, Germany). Fluorescence was induced by excitation at 480 nm through a narrow-band interference filter, and was detected after a second interference filter centered at 540 nm.

Each well of a white microtitration plate (Greiner Bio-One, Kremsmuenster, Austria) was pre-incubated with prionex 0.1 mg/ml (Pentapharm, Basel, Switzerlamd) and filled with 100 μl of buffer (140 mM NaCl, 20 mM HEPES, 1 mM EDTA for pH 7.0 and 140 mM NaCl, 20 mM MES, 1 mM EDTA for pH 4.0, 5.0, and 6.0) plus 100 μl of LUVs in the same solution. The final toxin concentrations were variable, and are reported in the text. After mixing vesicles and toxins, the release of calcein from the vesicles produced an increase in the fluorescence value F (due to dequenching of the dye into the external medium) which was resolved in time. The values reported are consecutive readings of the same well (taken every 16 s for 45 min). Spontaneous leakage of calcein was usually negligible; when present, it accounted for less than 10% release and it was subtracted on the basis of the spontaneous release observed in control experiments. In some cases fluorescence was measured in a spectrofluorometer (FluoroMax, Longjumeau, France). In these experiments, excitation was set at 494 nm and emission at 520 nm. In both cases, Maximum release was obtained by adding 1 mM Triton X-100 (Merck) (final concentration) and provided the fluorescence value F_{max}. The percentage of release, $R_{\%}$, was calculated as follows:

$$R_{\%} = (F_{fin} - F_{in})/(F_{max} - F_{in}) \times 100$$

where F_{in} and F_{fin} represent the initial and the final (steady-state) value of fluorescence before and after toxin addition. The experiments were carried out at room temperature.

Secondary Structure of Saporin Isoforms

Fourier transformed infrared spectroscopy. Fourier transformed infrared spectroscopy (FTIR) spectra were collected in the attenuated total reflection (ATR) configuration, as described in [Menestrina et al., 1999], on a Bio-Rad FTS 185 FTIR spectrometer equipped with a mercury–cadmium–tellurium detector and a KBr beamsplitter. Three samples were analyzed: SO6 and saporin mutants Δ1–20 and Δ239–253. For each experiment, 100 μg of protein were deposited in a thin layer on one side of a 10-reflection germanium crystal (45° cut), flushed with D_2O-saturated nitrogen and housed in a vertical ATR attachment (provided by Specac). A number of interferograms (64) were recorded, Fourier transformed and averaged. Absorption spectra in the region between 4000 and 500 cm^{-1} were obtained using a clean ATR crystal as the background, at a resolution of one point every 0.5 cm^{-1}. These spectra were

analyzed with the Bio-Rad WIN-IR software package based on GRAMS platform: They were at first corrected by subtracting the absorbance of residual H_2O and then processed to evaluate secondary structure content. Briefly, the amide I' band, between 1700 and 1600 cm^{-1}, was deconvoluted into a set of Lorentzians whose frequencies were assigned to a particular secondary structure in the standard way. The original spectrum was then curve-fitted with this set of Lorentzian components and the relative content of secondary structure elements was eventually estimated by dividing the areas of the individual peaks by the area of the whole amide I' band.

RESULTS AND DISCUSSION

The purification of saporin (SO6 and recombinant from *E. coli* cultures) and of the deletion mutants yielded preparations of >95% pure intact protein with negligible amounts of contaminants (<5%), as evaluated by SDS–PAGE and by immunoblotting (not shown). Protein samples were used in the assays described below.

Enzymatic Properties of SapΔ1–20 and SapΔ239–253 Mutants

To asses the enzymatic properties of the various proteins under study we used a test based on the release of adenine, which is in turn proportional to the N-glycosidase activity of the toxin preparations [Heisler et al., 2002]. A standard calibration curve was first established using known adenine concentrations and the enzymatic activity of the different forms of saporin was then titrated and compared on the standard curve. Results are summarized in Table I.

Reduced efficacy of sapΔ1–20 was further confirmed by kinetic assays (Fig. 1). Adenine release reaches a plateau at a much lower level as compared to wt isoforms of saporin or of sapΔ239–253, indicating that adenine amounts released by the enzymatic reaction are greatly affected. Moreover, calculated initial rate of adenine release values (V_0) (0.630 nmol/min) are also inferior to those of wt saporins or to sapΔ239–253, which range between 0.79 and 0.812 nmol/min (Table I).

Interaction of SapΔ1–20 and SapΔ239–253 Mutants With Artificial Membranes

The internalization and intracellular trafficking of a toxin are strongly dependent upon its ability to interact with the membranes of the intoxicated cell. Artificial membrane models mimicking the composition and behavior of cell membranes may, therefore, supply important information as regards internalization and routing mechanisms and possible perturbing factors influencing the cell intoxicating potential of different types of toxins. We have thus investigated the ability of the different saporin isoforms to induce the release of calcein entrapped in LUVs made with phosphatidic acid (PA), phosphatidylcoline (PC), or with an equimolar amount of PA and PC (PAPC). The net superficial charge is one electronic charge for PA and zero for PC phospholipids. Because the intracellular routing of internalized toxins involves compartimentalization within subcellular organelles at low pH, we have measured calcein release at pH values of 4.0, 5.0, and 6.0. Results are expressed as percent maximal release obtained by treating LUVs with the detergent Triton X-100. From summary Table II the following results can be evinced: (1) All forms of saporin interact only weakly with PC LUVs; (2) SO6 has an optimal effect of calcein release at pH 4.0 with PAPC LUVs (>90% release), at pH 5.0 with PA LUVs (>90%) and shows lower efficacy at pH 6.0 with PA LUVs (10–30%); (3) wt recombinant saporin shows a remarkable interaction with PAPC LUVs at

TABLE I. Comparison of Enzymatic Efficacy of Saporin and its Deletion Mutants

Toxin	Pmol adenin/pmol toxin/h	% Activity[a]	V_0 (nmol/min)
SO6	492 ± 1	98.4	0.79
Saporin$_{wt}$ (recombinant)	500 ± 1	100	0.81
SapΔ1–20	74 ± 1	14.8	0.63
SapΔ239–253	500 ± 0.5	100	0.80

[a]The enzymatic activity of full-length saporin$_{wt}$ (recombinant) is taken as 100%.

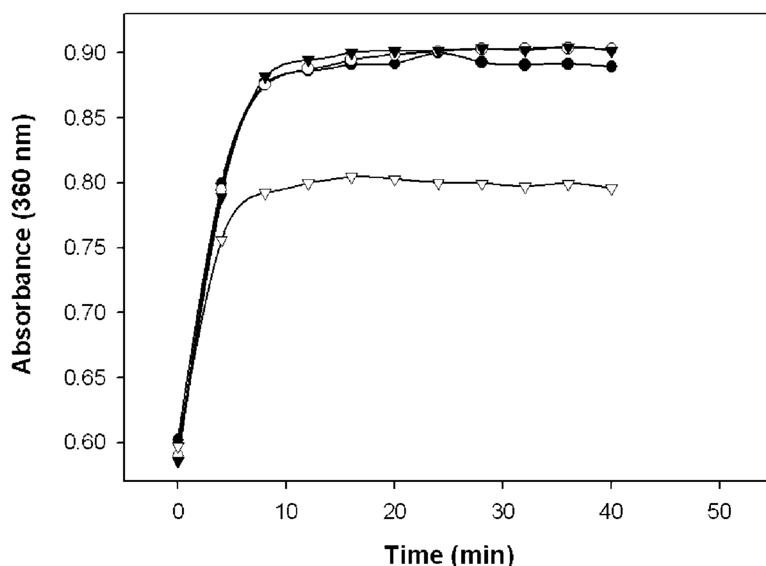

Fig. 1. The enzymatic activity of saporin isoforms was determined by measuring adenine release from the substrate denatured herring sperm DNA in a colorimetric assay. The kinetics of adenine release was monitored every 5 min with a microplate reader and the adsorbance measured at 360 nm. Symbols are as follows: Filled circles, SO6; empty circles, wt saporin, recombinant; filled triangles, sapΔ239–253; empty triangles, sapΔ1–20.

pH 4.0 (60–90% calceine release) but a modest effect on PAPC LUVs at pH 5.0 and on PA LUVs at pH 6.0 (10–30% and <10% calcein release, respectively). It appears, therefore, that SO6 and the wt isoform of recombinant origin may differ substantially as regards their ability to effect the release of calcein from LUVs of different composition (from this viewpoint calcein release from PA LUVs at pH 5.0 is particularly significant, Table II). In the deduced structure of SO6 alternative residues in positions 48 and 91 can be present [Savino et al., 2000], therefore, SO6 may exist in at least four different variants. Moreover, in our recombinant form of saporin a Phe is present in position 223 instead of the Val found in SO6 [Barthelemy et al., 1993]. Thus, although these variations are not likely to induce gross structural or behavioral differences we cannot rule out that at least some of the differences observed between our recombinant form of saporin and SO6 could be ascribed to them. In fact, the heterogenous composition of the sample obtained from seeds could explain the greater calcein release from LUVs treated with SO6 in some instances. Whether this be due to differences in the exposure of hydrophobic patches or to differences in the spatial orientation of protein microdomains remains to be investigated; (4) sapΔ239–253 behaves in a manner similar to wt recombinant saporin, that is, strong interaction with PAPC LUVs at pH 4.0 (60–90% calcein release), intermediate and modest interaction with PAPC LUVs at pH 5.0

TABLE II. Release of Calcein From LUVs of Different Composition

	PA			PAPC			PC		
Toxin (15 μg/ml)	pH 4.0	pH 5.0	pH 6.0	pH 4.0	pH 5.0	pH 6.0	pH 4.0	pH 5.0	pH 6.0
SO6	++[a]	+++	±	+++	+	±	–	–	–
$Saporin_{wt}$ (recombinant)	±	–	–	++	±	–	±	–	–
SapΔ1–20	++	+	–	+++	++	±	–	–	–
SapΔ239-253	±	±	–	++	+	–	–	–	–

[a]Values represent percentage of calceine release with respect to maximal release induced by treatment with Triton X-100; +++, R > 90%; ++, 60% <R < 90%; +, 30% < R < 60%; ±, 10% < R < 30%; –, R < 10%.

and with PA LUVs at pH 6.0 (30–60% and <10% calcein release respectively); (5) sapΔ1–20 displays efficient interaction with PAPC LUVs at pH 4.0 (>90% calcein release) and a considerable effect at pH 5.0 (60–90%). If the two deletion mutants are compared taking the calcein release effect of the wt recombinant form of saporin as 100% under the different conditions, it appears that although their interaction with PC LUVs is negligibly different from that of wt, sapΔ1–20 shows a considerable increase (144.7–329.7%) in the ability to effect calcein release from PA and PAPC LUVs. With respect to wt recombinant saporin the deletion mutant sapΔ239–253 displays instead similar effects on PA and PAPC LUVs (range of calcein release as a percent of wt recombinant saporin between 17.8 and +114%).

Cytotoxicity of SapΔ1–20 and SapΔ239–253 Mutants

To evaluate the effects of deletions on the cell intoxication potential of saporin we measured the cytotoxicity of the different saporin forms in a protein synthesis inhibition assay. The overall cytotoxicity in this case will be dependent upon several complex mechanisms, including binding to the cell surface, internalization kinetics, routing through subcellular compartments, interaction with intracellular membranes, and with molecular chaperones. Cytotoxicity was assayed in two cell lines of different histotype (Jurkat, a human leukemia, and DU145, prostate carcinoma). To rule out intrinsic resistance to ribosome inactivating mechanisms mediated by toxins and to compare the effects of various forms of saporin in cell lines of different histotype, the cytotoxic effects of whole heterodimeric ricin and of its catalytic subunit ricin A-chain were also studied in parallel. The results obtained in cytotoxicity assays are illustrated in Figure 2 and can be summarized as follows: (1) The cytotoxic effects of the wt isoforms of saporin are comparable in the same cell line, although DU145 cells show a greater resistance to intoxication by saporin. This however appears to be due to a general resistance to the effect of toxins, inasmuch as DU145 are less sensitive also to the effects of whole ricin (IC_{50} of 6.77×10^{-13} M in DU145 cells and of 1.96×10^{-13} M in Jurkat cells; results not shown) and in particular of its catalytic subunit ricin A-chain which, similar to saporin, lacks the ability to bind cell surface structures (Fig. 2); (2) both deletion mutants display a lower cytotoxicity in Jurkat and DU145 cells although, similar to the effect of wt saporin, the decrease is greater in Jurkat cells. In particular, however, sapΔ1–20 shows the greatest loss in cytotoxic activity, as compared to sapΔ239–253. Whereas the reduced cytotoxicity of sapΔ1–20 could be attributed mostly to a deficiency in catalytic activity, the diminished cell intoxicating effect of sapΔ239–253 might be due to more complex phenomena involving interaction with subcellular structures and membranes participating in intracellular routing and cytosol entry processes. In addition, our results with sapΔ1–20 allow to conclude that removal of a sizable domain at the N-terminus does not abolish saporin cytotoxicity confirming and extending results by Bagga et al. [2003a] who demonstrated that N-terminally point mutated saporin retained substantial cytotoxicity.

Saporin extracted from seeds of *S. officinalis* has been shown to bind to α_2-macroglobulin receptor, also called as low-density lipoprotein receptor-related protein (LRP) [Cavallaro et al., 1995]. It was recently found, however, that saporin toxicity is independent of the LRP expression in different cell lines, inasmuch as LRP-negative and LRP-defective cell lines showed the same sensitivity to saporin as LRP-positive cell lines [Bagga et al., 2003b]. Moreover, differences in cytotoxicity between LRP-positive and LRP-negative cell lines of different saporin isoforms were attributed solely to differences in catalytic activity [Bagga et al., 2003b]. It is, therefore, likely that also in our case the main differences could be attributed to mechanisms other than binding to specific cell surface receptors. Indeed, association of saporin to the cell line that we found least sensitive to saporin (i.e., DU145) was superior to the binding observed in a LRP-positive highly sensitive reference cell line (i.e., U937), as evaluated by indirect immunofluorescence and flow cytometry (not shown).

Secondary Structure of SapΔ1–20 and SapΔ239–253 Mutants

To investigate if the altered properties displayed by the deletion mutants of saporin could be due to substantial modifications of the protein structural organization, we evaluated secondary structures by means of infrared spectroscopy. To compare our observations with crystallographic data on SO6 [Savino et al.,

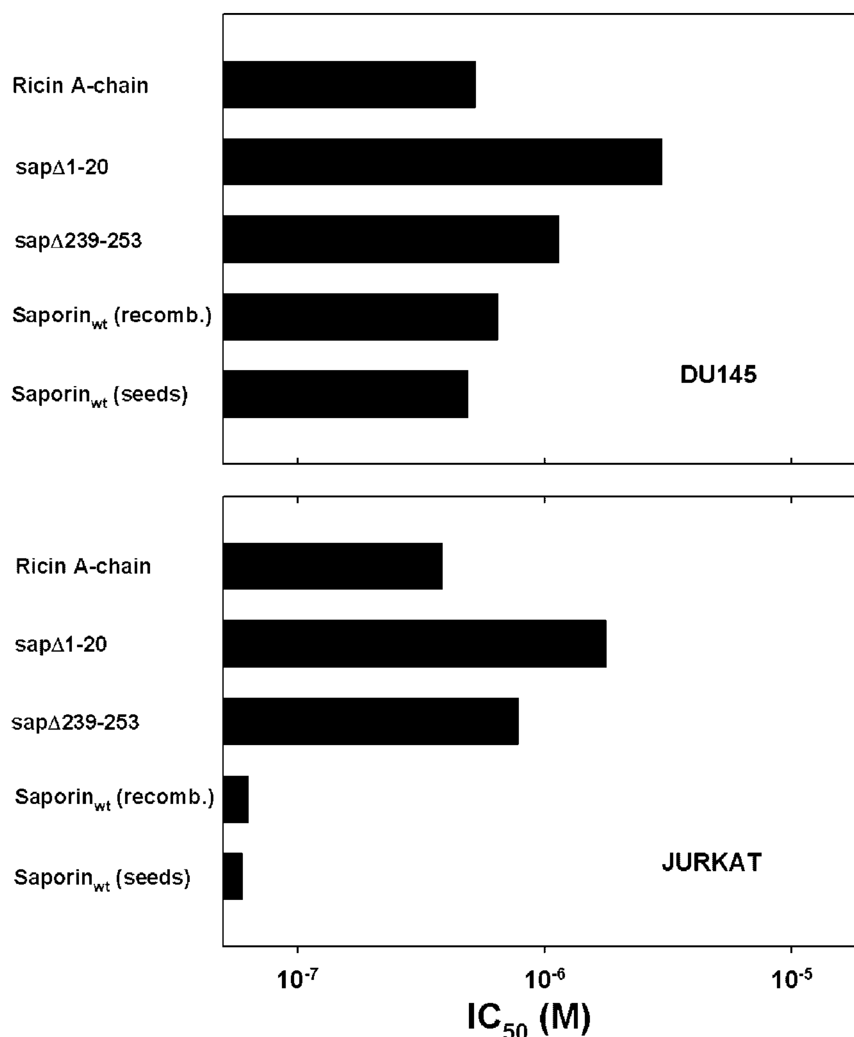

Fig. 2. The cytotoxicity of saporin forms was evaluated in a protein synthesis inhibition assay. The dose (mol/L) inhibiting 50% [^{14}C]-leucine incorporation (IC_{50}) is reported for the two cell lines (Jurkat and DU145) used in the assay. Standard deviation between replicates was <5% and is not reported in the Figure.

2000], we examined SO6 with the recombinant forms of saporin. The secondary structure composition of the different proteins was estimated by deconvolution and curve fitting with a sum of Lorentzians, which were assigned to different structural elements. According to [Susi and Byler, 1986] the following assignments were used: Band at 1675 ± 4 cm^{-1} or in the region 1640–1628 cm^{-1}, β sheet structure; band at 1654 ± 3 cm^{-1}, α helix. Data are reported in Table III. Based on [Savino et al., 2000] we predicted the distribution of different

TABLE III. Secondary Structure of Saporin Forms

	β-Sheet		α-Helix		Other	
Toxin	Expected[a]	Observed	Expected	Observed	Expected	Observed
SO6	17.79	20.73[b]	33.99	30.36	48.22	48.91
Saporin$_{wt}$ (recombinant)	17.79	27.93	33.99	25.88	48.22	46.19
SapΔ239–253	18.90	21.05	34.80	31.73	46.20	47.22
SapΔ1–20	16.73	48.68	31.75	14.29	51.50	37.03

[a]Estimated structure is according to Savino et al. [2000].
[b]Figures represent percent of total.

secondary structures and then compared our observations with the expected results. It can be noticed that, compared to all other saporin forms, the N-terminal deletion sapΔ1–20 mutant shows a sizeable increase in β-sheet structures with a drastic decrease in α-helices, considerably differing from predictions. It could be hypothesized that the deletion of the N-terminal fragment induces a profound rearrangement of saporin structure leading to an enhanced ability to interact with artificial membranes and also affecting the catalytic site. That the mutant sapΔ1–20 indeed displays a substantial alteration of secondary structure is also confirmed by tryptophan fluorescence analysis (not shown). Our results, therefore, indicate that diminished catalytic activity resulting from N-terminal deletions could depend on both the lack of the crucial Tyr 16 but also on considerable alterations of saporin structure. Likewise, an effect of N-terminal deletions on catalytic activity was observed in the case of ricin A-chain [May et al., 1989]. In this case, however, no data are available on possible structure rearrangements. Pittaluga et al. [2005] examined the effects of a 19 aminoacid deletion at the C-terminus of saporin and found that in such a deletion mutant the toxicity to bacterial cells is abolished and the enzymatic activity of saporin on polynucleotyde substrates (RNA or DNA) is severely impaired. In our case, we examined the effects of a deletion of only the last 15 C-terminal residues and we observed only a variable reduction of the cytotoxic properties of the molecule with negligible effects on its enzymatic properties in cell-free systems and on its overall structure. Moreover no major alteration of saporin secondary structure appears evident from our FTIR analysis after deletion of the 15 C-terminal aminoacids (Table III), although some variations were indeed observed with respect to full-length recombinant saporin. It is, therefore, possible that the further four aminoacids deleted by Pittaluga et al. [2005] are responsible for the effects that they observe, in particular the missing aminoacids could be important to stabilize saporin domains involved in the catalytic activity or in other functions necessary to enhance cytotoxicity in whole cells. As shown in Table III the observed structure of whole recombinant saporin differs somewhat from that of SO6. This aspect needs to be further elucidated. However, part of the considerations reported above to explain the different behavior of SO6 and recombinant saporin as regards their interaction with LUVs hold also in this case. In particular, the greater homogeneity of recombinant saporin may result in a secondary structure slightly different from what expected based on the data obtained with the more heterogeneous SO6.

Thus, collectively our data point to the fact that the N-terminal portion heavily influences both catalytic activity and interaction with lipid bilayers and can hardly be removed without profoundly affecting saporin functions. The C-terminal domain affects instead intracellular trafficking. It remains to be investigated whether further deletions in the C-terminal domain might allow to obtain a smaller saporin molecule retaining catalytic activity and cell intoxicating properties when delivered by cell-selective reagents. Additionally, new non-terminal deletions could be explored to evaluate whether size of saporin can be reduced while maintaining cytotoxic functions.

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