Type I Ribosome-Inactivating Proteins from *Saponaria officinalis*

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Abstract Saporins are ribosome-inactivating proteins (RIPs) extracted from different tissues of the soapwort plant (*Saponaria officinalis* L.). While the biosynthesis of these proteins and their roles in planta have received little attention, saporins have been extensively used for the production of targeted toxins for therapeutical and research applications. The biochemical features of one group of closely related saporin isoforms, collectively named SO6, have been characterized in considerable detail. In this chapter, we summarize available information on the saporin family of proteins, including their catalytic activity, 3D-structure, and biosynthetic and intoxication pathway(s), emphasizing the differences between the different family members and the characteristics that distinguish saporin from the catalytic subunit of the prototype Type II RIP ricin. The use of heterologous systems for the production of saporin and saporin-based chimeric toxins is also described.

1 Introduction

Ribosome-inactivating proteins (RIPs) are potent inhibitors of protein synthesis that act by catalytically depurinating an adenine residue (A4324 in rat) present in a conserved stem-loop region of 23/26/28S ribosomal RNA (rRNA), causing an irreversible arrest in protein synthesis (Endo and Tsurugi 1987, 1988; Endo et al. 1988; Hartley et al. 1991). The prototype RIP is the ricin AB dimer, whose

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biochemical features, catalytic activity, biosynthetic pathway, and intracellular transport have been studied in great detail. Ricin is synthesized as an inactive single-sized precursor that is transported to the protein storage vacuoles of castor bean endosperm cells and processed into disulphide-linked A (RTA) and B (RTB) chains (Butterworth and Lord 1983; Hiraiwa et al. 1997). The mechanism by which the ricin dimer intoxicates mammalian cells has also been thoroughly characterized (see chapter, "How Ricin Reaches its Target in the Cytosol of Mammalian Cells" by Spooner et al. in this volume) (Sandvig and van Deurs 2000; Lord et al. 2003). In contrast, little is known about the biosynthesis and trafficking of Type I (single chain) RIPs, their physiological function(s) in planta, and the mechanism(s) by which they reach the cytosol after uptake by mammalian cells.

The name saporin collectively identifies a family of RIPs that accumulate in different soapwort (*Saponaria officinalis* L.) tissues. Several cDNA and genomic clones coding for different members of the saporin family of proteins have been isolated, and individual isoforms (or mixtures of closely related isoforms) have been purified. The three-dimensional structure of one isoform has been solved, and the enzymatic activity of individual family members has been studied in some detail. While some characteristics of the saporin proteins, such as key catalytic residues and overall three-dimensional fold, are shared with RTA, certain biochemical and functional properties clearly diverge. In this chapter, we will describe the principal characteristics of the saporin protein family, highlighting both the differences between different family members and the specific features that distinguish these proteins from RTA and from other Type I RIPs.

2 Saporin Multigene Family and Saporin Isoforms

Saporins are encoded by a small multigene family (Fordham-Skelton et al. 1990; Barthelemy et al. 1993). While saporins are often designated on the basis of the tissue of origin and the number of the chromatographic peak in ion-exchange chromatography, it should be stressed that these chromatographic peaks can contain two or more closely related isoforms, and that the use of different purification procedures implies that peaks having the same or similar names cannot be assumed to contain an identical set of proteins. In addition, some recombinant proteins have in some instances been given a name similar to the one used for chromatographic peaks. Thus, saporin nomenclature is somewhat confusing, and attention must be paid when comparing data from different sources.

The presence of multiple RIP isoform has been reported for different members of the Caryophyllaceae family of plants, such as *Dianthus caryophyllus* (Stirpe et al. 1981) (a plant belonging to the same subfamily as *S. officinalis)*, *Lychnis chalcedonica* (Bolognesi et al. 1990), and *Petrocoptis glaucifolia* (Arias et al. 1994). The tissue distribution of dianthin 30 and 32 in *D. caryophyllus* was investigated by the use of anti-dianthin antibodies; while dianthin 30 was found throughout the plant, seeds included, dianthin 32 was detected only in leaves and

growing shoots (Reisbig and Bruland 1983). Both isoforms accumulate in old tissues, where they represent between 1 and 3% of the total extractable protein.

The tissue distribution of saporin, like that of dianthin, contrasts with that of the RIPs present in the Gramineae family (Coleman and Roberts 1982), or in *Ricinus communis* (Tregear and Roberts 1992), where the RIPs are apparently confined to the seeds. When translation inhibitory activity was monitored in different soapwort tissues, it was detected in all those that were examined (leaves, stems, roots, flowers, and fruits), except immature seeds (Ferreras et al. 1993). A high level of activity was found in roots and mature seeds, while old and young leaves contained similar activity. The expression of saporin has also been studied in callus, cell suspensions, and root cultures from soapwort explants (Di Cola et al. 1997). High specific activity was found in callus extracts, while lower levels were present in root extracts. In addition, culture senescence and abscisic acid were found to induce saporin production in cultures of soapwort roots (Di Cola et al. 1999). These results suggest that callus and cell cultures may be a suitable model system to study saporin biosynthesis and biological function.

After fractionation of soapwort plant extracts, most of the translation inhibitory activity was found to be associated with three chromatographic peaks in seeds, two in leaves, and three in roots (Stirpe et al. 1983; Ferreras et al. 1993). N-terminal sequencing suggested that saporins present in these chromatographic peaks could be divided into three groups, each group being specific to one of the three organs, with the exception of one root isoform (R2) that has an N-terminal sequence similar to the ones of the two leaf isoforms (L1 and L2) (Ferreras et al. 1993). Notwithstanding the identical N-terminal sequence, saporin L1 and R2 have distinct biochemical properties. Two out of three root saporins (R1 and R3) were reported to be glycosylated and to contain cysteine residues, an amino acid which is absent in all other saporin isoforms (Ferreras et al. 1993).

Two of the three (5, 6, and 9) major peaks of activity identified in seed extracts have been characterized in detail (Stirpe et al. 1983). SO6 saporin represents the major peak (peak 6) and constitutes about 7% of the total seed protein. Direct sequencing of the protein revealed heterogeneity at two positions, with either aspartic or glutamic acid in position 48, and either lysine or arginine present in position 91 (Maras et al. 1990; Barra et al. 1991). These data indicate that the SO6 peak contains a set of closely related saporin isoforms. Indeed, RP-HPLC analysis confirmed the presence of atleast three different isoforms in SO6 preparations (Fabbrini et al. 1997a).

The primary structure of SO9 saporin (peak 9) has also been determined (Di Maro et al. 2001). The protein contains four histidine residues, an amino acid which is absent in all the other known seed isoforms, and presents 22 amino acidic substitutions when compared to SO6. No heterogeneity was found in this case, indicating that the SO9 peak contains a single saporin isoform. A preliminary crystallographic characterization of this protein has also been reported (Kumar et al. 1999).

The first DNA sequence coding for a saporin isoform was isolated from a leaf cDNA library (Benatti et al. 1989). Comparison with the sequence of

seed-extracted SO6 suggests that the polypeptide encoded by this cDNA clone contains both a signal peptide and a C-terminal extension. The predicted mature protein contains 11 amino acid differences when compared to SO6.

Three genomic clones, termed Sap2, Sap3, and Sap4, were also successively isolated (Fordham-Skelton et al. 1990, 1991). Two of them (Sap3 and Sap4) were truncated, while Sap2 was found to encode a full-length saporin precursor. Comparison of the sequence encoded by the Sap2 clone with the one of SO6 reveals again the presence of a signal peptide for insertion in the endoplasmic reticulum (ER) and of the C-terminal propeptide that must be removed to generate the native SO6 C-terminus (Fig. 1b). After removal of the signal peptide and of a C-terminal propeptide, the Sap2-encoded protein would be identical to one of the four putative isoforms potentially present in the SO6 peak.

Subsequently, five further partial clones (numbered 1–5) were isolated by PCR amplification of soapwort genomic DNA (Barthelemy et al. 1993). Of the encoded proteins, two are similar to the one encoded by the leaf cDNA clone (Benatti et al. 1989) while the others encode SO6 or SO6-like polypeptides. Three of these isoforms showed identical translation inhibition activity when recombinantly expressed in *E. coli* (Fabbrini et al. 1997a). In contrast, the protein encoded by the leaf cDNA clone (Benatti et al. 1989), also termed saporin-C, was 10-fold less active in the same assays. Consistently, the sequence of clone 5 described by Barthelemy et al. (1993) codes for a protein which is closely related to saporin-C and which is less active than the product of clone 2, which codes for one of the components present in the SO6 peak (Bagga et al. 2003a).

A few further cDNA sequences encoding saporin polypeptides have been deposited in the DNA data banks.

3 Saporin Biochemical Features

3.1 Saporin Structure

SO6 polypeptides are composed of 253 residues, corresponding to a molecular weight of ~28,600 Da (Maras et al. 1990). The proteins contain a net positive charge with an isoelectric point (pI) above 9.5 (Lappi et al. 1985; Di Maro et al. 2001). Lysine residues, which represent ~9% of the total, are particularly abundant. The SO6 proteins are also characterized by high resistance to chemical denaturation and proteolytic degradation in vitro (Santanché et al. 1997). Deletion of the first 20 amino acids has been shown to drastically affect saporin folding (Bonini et al. 2006), while deletion of the last 19 amino acids has a detrimental effect on catalytic activity (Pittaluga et al. 2005).

Despite the structural similarity between saporin, RTA, and other Type I RIPs, sequence identity is low: only 62 residues (about 22%) are conserved between RTA and SO6, and 44 residues (about 15%) between the latter and trichosanthin (TC).

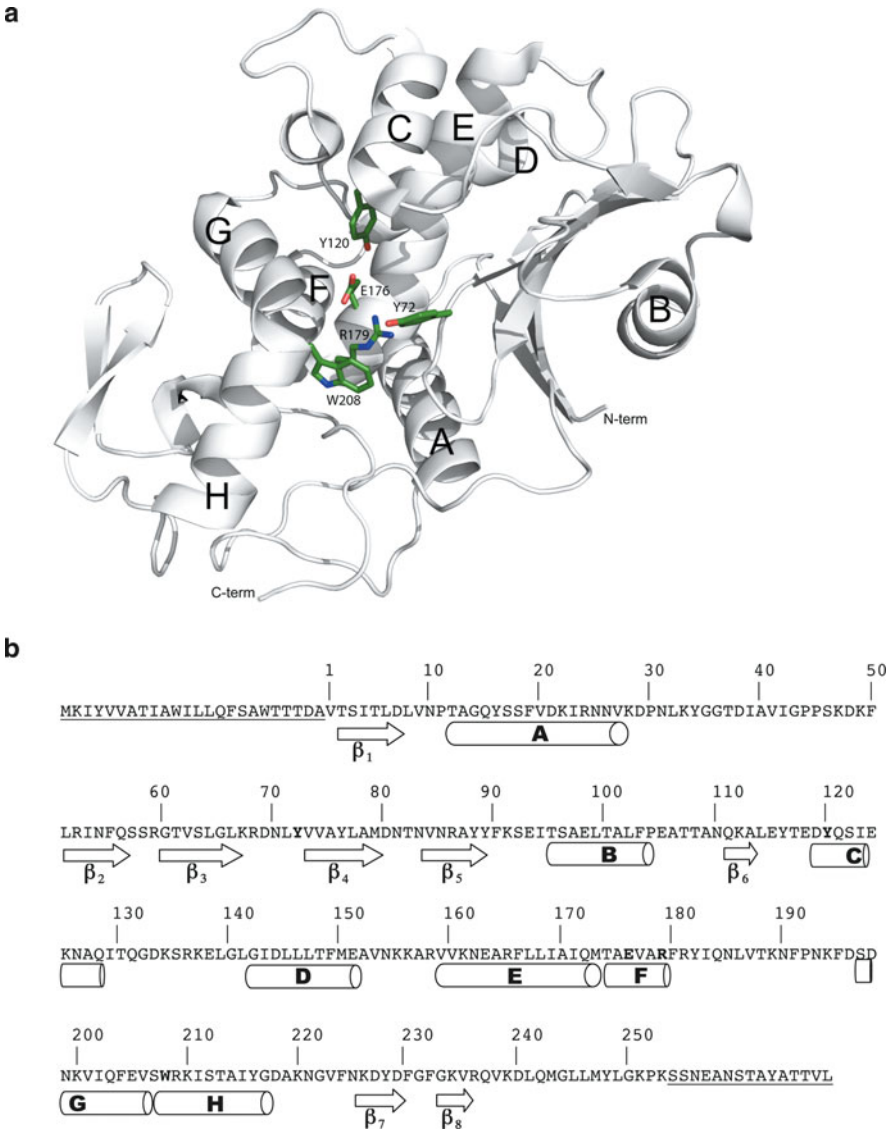

Fig. 1 Overall 3D-structure of saporin. (**a**) Ribbon representation of SO6 structure (PDB code: 1qi7) in which the secondary structure elements are shown. The five conserved residues in the active site (Tyr 72, Tyr 120, Glu 176, Arg 179, and Trp 208) are shown in *stick representation*. (**b**) Amino acid sequence of a putative SO6 saporin precursor, as deduced by the DNA sequence of the Sap2 clone (Fordham-Skelton et al. 1991). Helices are shown as *cylinders* and are named following the canonical RIP nomenclature. Strands are shown as *arrows*. The conserved active site residues are shown in *bold*. The N-terminal signal peptide and the C-terminal propeptide sequences are *underlined*. Numbering starts with the first amino acid of the mature protein

On the contrary, a high degree of sequence identity (around 80%) is found between saporin and dianthin, both of which are synthesized by plants belonging to the subfamily Silenoideae of the Caryophyllaceae family.

The three-dimensional structures of different Type I RIPs including momordin (Husain et al. 1994), pokeweed antiviral protein (PAP) (Monzingo et al. 1993), TC (Zhou et al. 1994), gelonin (Hosur et al. 1995) and, more recently, dianthin (Fermani et al. 2005) have been determined. SO6 has been crystallized (Savino et al. 1998) and the crystal structure resolved (Savino et al. 2000). Saporin shares with other Type I RIPs and with RTA a common "RIP fold" characterized by the presence of two major domains: an N-terminal domain which is predominantly β-stranded, and a C-terminal domain that is predominantly α-helical (Figs. 1 and 2). Insertions and deletions as compared to PAP, momordin, and RTA lie mainly in the random coil regions. Most of the secondary structural elements are comparable between saporin and other Type I RIPs. The deviations are seen mainly in some surface-located loop regions, particularly: (1) between strands β_4 and β_5 (residues 79–85), (2) helix B and the loop connecting this helix to strand β_6 (residues 95–109), and (3) between helices C and D (residues 128–134). Figure 2 shows, as an example, a superimposition of the SO6 and RTA structures. Interestingly, the loop connecting strands β_7 and β_8 located at the C-terminal domain, whose length is variable among RIPs, is very short in SO6 (only three residues), and in dianthin 30 (Fermani et al. 2005), but is longer in PAP and RTA. This region contains three lysine residues (220, 226 and 234), which seem to be involved in the molecular recognition of the ribosome. The reduced length of this loop could determine an increased accessibility to the substrate for both saporin and dianthin. The catalytic cleft is almost perfectly superimposable in all RIPs, including saporin, except for

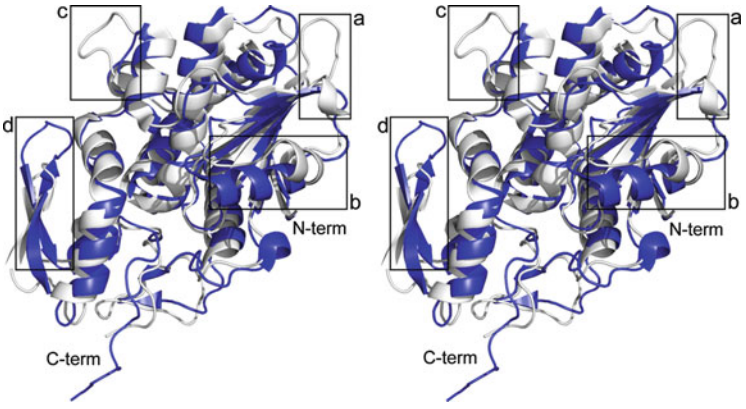

Fig. 2 Structural comparison based on superimposition of secondary structure elements of SO6 (PDB code: 1qi7, *white*) with the A chain of ricin (PDB code: 1j1m, *black*), stereoview. The regions with the largest deviation are included in *boxes* and *labeled*: (**a**) loop between strands β_4 and β_5 (residues 79–85), (**b**) helix B and the loop connecting this helix to β_6 (residues 95–109), (**c**) CD loop (residues 128–134) and (**d**) loop connecting strands β_7 and β_8 (residues 230–233)

the orientation of one of the two tyrosine residues (Tyr 72 in saporin) that are involved in the interaction with the target adenine (Fermani et al. 2009).

3.2 Saporin Catalytic Activity

Saporin is an RNA *N*-glycosidase that removes a specific adenine residue (A4324 in rat) located in the highly conserved GAGA-tetraloop, also termed the α-sarcin/ricin-loop, present in 23/26/28S rRNA (Endo et al. 1988). Like RTA (Endo and Tsurugi 1987), SO6 has been found to release a single adenine from 80S ribosomes (Sturm et al. 2009). However, removal of a second adenine residue has also been reported (Fermani et al. 2009).

Typically, saporin SO6 preparations and recombinant SO6 proteins are found to inhibit translation in a rabbit reticulocyte lysate with an IC_{50} in the low picomolar range (Ferreras et al. 1993; Fabbrini et al. 1997a; Bagga et al. 2003a, b; Pittaluga et al. 2005). While *E. coli* ribosomes are more resistant than mammalian ribosomes to the action of different saporins (Ferreras et al. 1993; Girbés et al. 1993), the difficulties in expressing various saporin isoforms in *E. coli* suggest that they are toxic to this host (Fabbrini et al. 1997a). Indeed dianthin 32, which is highly similar to SO6 saporin, was found to be both ~500 times more active on yeast than on *E. coli* ribosomes, and specifically to depurinate 23S rRNA at a site which is equivalent to A4324 in rat 28S rRNA (Hartley et al. 1991).

Different saporin preparations have been found to be active in inhibiting protein synthesis from plant (*Vicia sativa*, *Cucumis sativus*, wheat germ) ribosomes (Ferreras et al. 1993). In particular, a chromatographic fraction named saporin 5 was shown to depurinate *V. sativa* ribosomes at a site corresponding to A4324 in rat 28S RNA, although other depurination sites were evident as well (Iglesias et al. 1993). We have also found that saporin expression is highly toxic to tobacco protoplasts and that this toxicity depends on the presence of an intact active site (our unpublished observation).

Several saporin fractions have been shown to depurinate naked nucleic acids such as viral genomic RNA, herring sperm DNA, rRNA, and poly(A) RNA, and to release more that 1 mole of adenine per mole of ribosomes, thus possessing polynucleotide:adenosine glycosidase (PNAG) activity (Barbieri et al. 1992, 1994, 1997). This activity has been characterized in detail for the saporin fraction L1. The protein was found to inhibit translation in a reticulocyte lysate system with an IC_{50} of ~45 pM (Sturm et al. 2009) and to release ~6.5 adenines from rat liver ribosomes before 50% inhibition was observed in an in vitro assay with poly(U) transcript (Barbieri et al. 1996). Under appropriate conditions, saporin L1 was found to depurinate DNA extensively and released adenine from all adenine-containing polynucleotides tested. Characterization of the kinetic parameters indicated that poly(A) RNA depurination proceeds with a K_m of 639 ± 32 μM and a k_{cat} of 61 ± 1 min^{-1} at pH 7.8 and 25°C. The catalytic efficiency of L1 on this substrate thus appears to be considerably lower compared to the action of a typical

RIP, such as ricin A chain, on intact rat ribosomes, which has been reported to occur with a K_m of 2.6 μM and a k_{cat} of 1,777 min^{-1} (Endo and Tsurugi 1988). Determination of the kinetics of ribosome depurination at different sites in relation to the inhibition of protein synthesis will be required to understand the mechanism of 80S ribosome inactivation by saporin L1.

In addition to saporin L1, other members of the saporin family have been shown to be endowed with PNAG activity. All tested saporins were active on herring sperm DNA at pH 4.0, while activity on poly(A) RNA, rRNA, and viral RNA varied widely between different members of the family. Two leaf saporin fractions and one root fraction (all sharing similar N-terminal sequences) appeared to be generally more active than other isoforms on poly(A) RNA, rRNA, and viral RNA (Barbieri et al. 1997). The activity of seed-extracted SO6 was found to vary between different batches or experiments when poly(A) or viral RNAs were used as substrates (Barbieri et al. 1997; Fermani et al. 2009). Recombinant saporins were instead essentially inactive on poly(A) and viral RNAs, but active on rRNA at pH 4.0 (Barbieri et al. 1997).

The biological significance of the activity against rRNA at sites different from the one attacked by ricin, and on substrates other than the ribosome (DNA, viral RNA, poly(A) RNA), remains to be established. When rRNA was extracted from mammalian cells treated with an SO6 saporin fraction, rRNA was found to be depurinated at a single site most likely corresponding to the ricin target (Vago et al. 2005). Analysis of the in vivo activity of other saporin fractions (such as fraction L1) will be required to assess whether multiple depurination plays any major role in the intoxication process.

In addition to these depurinating activities, saporin has been proposed to have DNase-like activities (Roncuzzi and Gasperi-Campani 1996; Ghosh and Batra 2006). However, several lines of evidence indicate that the DNase activity associated with saporin (and with other RIPs) may be due to contamination (Day et al. 1998; Barbieri et al. 2000; Peumans et al. 2001; Lombardi et al. 2010).

3.3 Residues Important for the Catalytic Activity

The active site of the SO6 protein includes a number of residues that are conserved in the RIP family of proteins: Tyr 72, Tyr 120, Glu 176, Arg 179, and Trp 208 (Fig. 1). The role of these residues in the catalytic activity of a component of the SO6 peak (named saporin 6), has been systematically investigated by mutating them to Ala (Bagga et al. 2003b). Mutating Tyr 72 had a stronger impact on saporin 6 catalytic activity than mutating Tyr 120. This is similar to what has also been observed for the corresponding tyrosine residues in RTA where mutation of Tyr 80 and Tyr 123 reduced RTA catalytic activity 160- and 70-fold, respectively (Monzingo and Robertus 1992).

Both Glu 176 and Arg 179 are thought to be directly involved in saporin 6 catalysis. However, while the Glu 176 mutant was 20-fold less active than

wild-type saporin 6 in inhibiting translation in a reticulocyte lysate, the Arg 179 mutant was 200-fold less active. In RTA, the same mutation in Glu 177 facilitates the nearby Glu 208 to move into the active site, fulfiling the role of Glu 177 (Frankel et al. 1990; Kim et al. 1992). It has therefore been proposed that, in the corresponding saporin 6 mutant, Glu 205 occupies the position of the mutated Glu 176, but that the carboxylate of Glu 205 provides less stabilization to the oxycarbonium ion transition state than Glu 176 (Bagga et al. 2003b). Interestingly, a Glu 176 change to a lysine (a residue bearing an opposite charge) led to a more drastic increase in the IC_{50} in an in vitro assay (Pittaluga et al. 2005).

A complete loss of in vivo toxicity was obtained by mutating both Glu 176 and Arg 179 into lysine and glutamine, respectively. This double mutant (termed KQ) is devoid of the detrimental effects associated with RIP expression in several different hosts (Zarovni et al. 2007; Lombardi et al. 2010). Intriguingly, no correlation between the in vitro enzymatic activity and cytotoxicity was reported for a saporin mutant at Trp 208 (Bagga et al. 2003b) while this same residue was seen to be crucial for the structural integrity of PAP (Rajamohan et al. 2000).

Tyr 16 and Arg 24 are two other invariant residues lying outside the active site and present among various RIPs, but whereas mutation at Arg 24 did not have any effect on the enzymatic activity of saporin 6, mutation at Tyr 16 resulted in a complete loss of the RNA *N*-glycosidase activity (Bagga et al. 2003b). In contrast, deletion of residues 21–23 of RTA (including a tyrosine residue equivalent to Tyr 16 in saporin 6) did not affect the functional activity of the protein (Morris and Wool 1992), while mutation of Tyr 14 to Phe in TC resulted in a relatively small (5-fold) decrease in RIP activity (Shaw et al. 1997). The basis of these differential effects remains to be clarified.

A contribution from Asn 162 of saporin 6 has been highlighted by a study in which the catalytic activity of two saporin isoforms, encoded by two different genomic clones (corresponding in sequence to clone 2 and clone 5 described by Barthelemy et al. 1993) has been compared (Ghosh and Batra 2006). The product of clone 2 (saporin 6) is about 10-fold more active than the product of clone 5 (saporin 5) in inhibiting protein synthesis (Bagga et al. 2003a). Among the 12 amino acid differences between the two proteins, six are conservative in nature. Five other amino acid differences were found to be irrelevant, but when Asn 162 of saporin 6 was replaced with Asp (the amino acid found at that position in saporin 5), the IC_{50} of the protein in an in vitro translation system increased $\sim$10-fold. Asn 162 is proximal to a set of hydrophobic residues placed on a neighboring helix, and the introduction of a negative charge at this position has been proposed to affect the stability of the active site (Ghosh and Batra 2006).

3.4 Interaction with the Ribosome

Although RTA can depurinate naked rRNA, the k_{cat} value of such a reaction is 10^5-fold lower than that for rRNA associated with proteins in the ribosome

(Endo and Tsurugi 1988). This suggests that the interaction between the RIP and the ribosomal proteins is essential to achieve optimal enzymatic activity. RTA can be cross-linked to mammalian ribosomal proteins L9 and L10e (Vater et al. 1995), whereas PAP gains access to the ribosome by recognizing L3 (Hudak et al. 1999). In eukaryotic ribosomes, P0, P1, and P2 proteins form a pentameric P-complex (P0 $(P1)_2(P2)_2$) which constitutes the so-called ribosomal stalk (Tchorzewski 2002). The P-complex docks onto the ribosome through an interaction with 28S rRNA and forms the GTPase-associated center for binding elongation factors during protein synthesis (Uchiumi and Kominami 1992). The ribosomal stalk has been implicated in the binding of TC, RTA, and Shiga-like toxin 1 A_1 chain to the ribosome (Chan et al. 2007; McCluskey et al. 2008). In the case of TC, three key basic residues (Lys 173, Arg 174 and Lys 177) located in the C-terminal domain are involved in P2 binding.

Chemical cross-linking studies suggest that at least one 30 kDa ribosomal protein from the 60S yeast ribosomal subunit comes into contact with saporin (Ippoliti et al. 1992). Savino and coworkers further studied the molecular interaction(s) between SO6 and the yeast ribosome by differential chemical modifications and identified a contact surface within the C-terminal region of saporin which includes three lysine residues in positions 220, 226, and 234 (Savino et al. 2000).

A negative electrostatic potential, arising from both the negatively charged phosphodiester backbone and from conserved solvent-exposed acidic patches on the ribosomal proteins, covers much of the ribosomal surface (Baker et al. 2001). The net positive charge of saporin and its high content of basic residues are likely to be critical for the recognition of the ribosomal surface. In RTA, a set of arginine residues in the region of the active site are involved in electrostatic interaction with the phosphodiester backbone of the sarcin–ricin loop (Monzingo and Robertus 1992; Marsden et al. 2004). Both RTA- and saporin-catalyzed rRNA modification shows a net dependence on salt and ion concentrations, indicating that these toxins exploit multiple electrostatic interactions with the target ribosomes (Korennykh et al. 2007). These recent studies confirm that Coulomb interactions play a crucial role in helping saporin (and other RIPs) in finding their ribosomal target sites, and may explain how RIPs can operate on the ribosome with k_{cat} and K_m exceeding their basal encounter frequency by more than an order of magnitude.

3.5 Saporin Inhibitors

During the last few years there has been an increasing interest in identifying small molecules which may act as inhibitors of RIPs for diagnostics, as antidotes to poisoning, or to avoid side-effects following administration of immunotoxin therapies, such as the post-therapy vascular leak syndrome (Baluna and Vitetta 1997). Although RNA aptamers have been considered as potential inhibitors of RIPs (Hesselberth et al. 2000), previous studies identified small ring molecules, such

as formycin, that interfered with ricin enzymatic activity (Yan et al. 1998). The 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP) adenine analog was also shown to inhibit different RIPs to several different extents; whereas it was ineffective on ricin, it had some effect on Shiga toxin and SO6 (Brigotti et al. 2000), indicating that RIPs differ in their ability to fit adenine analogs within the active-site cleft, presumably due to local sequence variability. Interestingly, linear, cyclic, and stem–loop oligonucleotides mimicking the catalytic transition state showed potent inhibitory effect on the leaf saporin L1 isoform but not on seed-extracted saporin SO6 (Sturm et al. 2009).

4 Saporin Trafficking and Toxicity in Eukaryotic Cells

4.1 Subcellular Distribution of Saporin Isoforms in Soapwort Tissues

RIPs have been found to accumulate both intracellularly and in the apoplast. For instance, ricin accumulates in the matrix of vacuolar protein bodies (Tully and Beevers 1976; Youle and Huang 1976; Lord et al. 1994), while PAP is deposited extracellularly in leaves (Ready et al. 1986). A detailed study of the subcellular localization of saporin in soapwort seeds and leaf tissue has been performed using immunogold labeling techniques and anti-SO6 antibodies (Carzaniga et al. 1994). During seed development, saporin was found to accumulate in the perisperm, a maternal tissue derived from the nucellus that represents the major storage tissue in the Caryophyllaceae. In the perisperm, saporin was localized both intracellularly and extracellularly. In developing seeds, saporin was immunolocalized to the ER and cytoplasmic vesicles, and accumulated within the large central vacuole, either in small isolated deposits or in large protein aggregates. Outside perisperm cells, saporin was found in the intercellular spaces and the paramural region, between the plasmalemma and the primary cell wall, but was not detected within the cell wall matrix. Interestingly, saporin was also found within the residue of pollen-tube exudates. No accumulation of the toxin was observed within the embryo in either developing or mature seeds, indicating that expression of saporin genes is strictly tissue-specific. While these data indicate that saporin accumulates at several sites in the perisperm, it remains unclear whether this distribution is due to the expression of differentially targeted isoforms, to the presence of inefficient vacuolar targeting signals, or to both of these factors.

In leaves, immunolocalization using an anti-SO6 antibody showed saporin to be associated with the intercellular spaces within the chlorenchima, while no labeling was observed within the protoplasm (Carzaniga et al. 1994). However, the lack of an intracellular signal may be due to the presence of saporin isoforms that are not recognized by the anti-SO6 serum. More recently, a study based on mass spectrometry analyses and N-terminal sequencing of the apoplastic and intracellular leaf

isoforms identified saporin-L1 as the most abundant saporin vacuolar isoform, while the apoplastic forms were more related to seed-like isoforms (De Angelis et al. 2001).

4.2 Saporin Biosynthesis and Role in Planta

Signal peptide-mediated targeting of saporin precursors to the ER and segregation within the secretory pathway is likely to be essential to protect soapwort ribosomes from inactivation. Indeed, several saporin encoding cDNAs and genomic clones have been shown to encode a precursor form containing an N-terminal signal peptide (Benatti et al. 1989; Fordham-Skelton et al. 1991). In addition to an N-terminal signal peptide, different saporin isoforms may also contain a C-terminal propeptide. Conclusive evidence for the presence of both a signal peptide and a C-terminal extension has been obtained in the case of SO6. One of the clones (Sap2) described in Fordham-Skelton et al. (1991) encodes a protein whose sequence corresponds to one of the four possible components of the SO6 peak. Comparison of the predicted amino acid sequence of the Sap2 genomic clone with the one of the SO6 protein (Maras et al. 1990; Savino et al. 1998) reveals that SO6 is synthesized as a precursor with a 24 amino acid signal peptide and a 15 amino acid C-terminal extension that presents some similarity with C-terminal propeptides known to contain a vacuolar targeting signal (Vitale and Hinz 2005). Apoplastic saporin isolated from leaf tissue has a molecular weight similar to that of the seed SO6 protein, thus suggesting that an SO6-like protein is expressed in leaves, and that it accumulates in the apoplast. Indeed, cDNA clones encoding SO6-like proteins have been isolated starting from leaf mRNA (Benatti et al. 1991; accession number DQ105520). These results raised the possibility that SO6 proteins are, at least in part, responsible for the extracellular accumulation of saporin in perisperm tissue. If this was the case, other isoforms must be responsible for the accumulation of saporin in the vacuoles of perisperm cells. Alternatively, an SO6 vacuolar targeting signal may be more effective in seeds than in leaf tissue. Indeed, partial targeting of a seed saporin isoform to the vacuole is observed when the protein is expressed in tobacco protoplasts (our unpublished observation).

Saporin L1 has been shown to accumulate intracellularly in soapwort leaves (De Angelis et al. 2001), but the sequence responsible for the intracellular retention of this protein remains unknown. The isolation of a cDNA clone (accession number DQ105519) encoding a protein whose N-terminus (after signal peptide removal) corresponds to that of saporin L1 has been reported. A comparison between the molecular mass of intracellular saporin L1 (De Angelis et al. 2001) and of that predicted by this cDNA clone suggests that L1 saporin is also synthesized as a precursor containing a C-terminal propeptide.

The biological function of saporins *in planta* is still unknown. While the ability of different RIPs (including saporin SO6 and SO9 fractions) to inhibit viral replication is well documented (Stirpe et al. 1983; Taylor et al. 1994), the

underlying mechanism(s) remains uncertain. According to the local suicide hypothesis (Ready et al. 1986; Kataoka et al. 1992), cells in which the plasma membrane is transiently breached by a virus vector would permit entry of apoplast-located toxin and be killed through ribosome inactivation. This localized cell death would block both replication and spread of the virus throughout the plant. In this model, preaccumulation of the RIP in the apoplast may be crucial. However, this model of toxin action has been criticized, since protein synthesis in damaged cells would stop anyway, independent from RIP action (Peumans et al. 2001). An intriguing, alternative possibility is that physiological mechanisms might be in place to regulate access of a particular RIP to cytosolic ribosomes, so that protein synthesis is affected only when the cell becomes infected. The observation that Iris RIPs protect plants from local but not from systemic infection indicates that their antiviral activity is effective only in initially infected cells (Vandenbussche et al. 2004). Conceivably, specific signals in these cells may lead to a change in the subcellular localization of the stored toxin, or to the degradation of a putative RIP inhibitor (Desvoyes et al. 1997). The elucidation of the mechanisms that avoid self-intoxication of soapwort tissues and the development of methods to monitor saporin subcellular localization and ribosome depurination during viral infection may help our understanding of the role played by Type I RIPs in plants.

Saporin has also been reported to be toxic to two Coleoptera species (Gatehouse et al. 1989), and a direct effect of the RIP on insects remains an interesting possibility.

4.3 Intoxication Pathways in Mammalian Cells

The intracellular transport of RIPs after internalization by mammalian cells has been studied in great detail in the case of ricin (see the chapter, "How Ricin Reaches its Target in the Cytosol of Mammalian Cells" by Spooner et al. in this volume). Briefly, ricin enters target mammalian cells by receptor-mediated endocytosis and undergoes retrograde transport to the ER where its catalytic A chain (RTA) is reductively separated from the cell-binding B chain (Spooner et al. 2004). Free RTA then enters the cytosol where it inactivates ribosomes. In order to cross the ER membrane, RTA mimics ER-associated degradation (ERAD) substrates, probably escaping proteasomal degradation thanks to its paucity of lysine residues. Once in the cytosol, RTA interacts with Hsc70 chaperones, with its destiny (folding or degradation) then depending on the cochaperones that regulate Hsc70 activity (Spooner et al. 2008).

Less attention has been paid to the pathway followed by saporin, and Type I RIPs in general, in mammalian cells during the intoxication process. Saporin cytotoxicity varies dramatically between different mammalian cell lines, with the concentrations inhibiting protein synthesis by 50% (IC_{50}) ranging from the nanomolar to the micromolar range, thus spanning three orders of magnitude (Cavallaro et al. 1995; Bagga et al. 2003b). Different lines of evidence indicate that members of the LDL family of proteins are involved in the initial stages of

saporin endocytosis. The LDL receptor family includes seven closely related family members: LDLR, the very-low-density lipoprotein (VLDL) receptor, apoE receptor 2, multiple epidermal growth factor-like domains 7 (MEGF7), glycoprotein 330 (gp330/megalin/LRP2), LRP1, and LRP1B. These proteins have been shown to be promiscuous in ligand binding (Lillis et al. 2008). The family also includes more distantly related members, such as LRP5, LRP6, and SorLa/LRP11. One of these receptor proteins, the α_2-macroglobulin receptor/low-density lipoprotein receptor-related protein (LRP1), was shown to bind saporin in vitro (Cavallaro et al. 1995; Fabbrini et al. 1997a) and is able to mediate saporin internalization in human monocytes and fibroblasts (Conese et al. 1995; Fabbrini et al. 1997b). Evidence that LRP1 mediates saporin endocytosis has also been obtained in the case of human promyelocytic U937 cells, where the downregulation of LRP nicely paralleled resistance to saporin and to a urokinase–saporin conjugate (Conese et al. 1995). Specific displacement of iodinated LRP1-receptor associated protein (RAP) with saporin in these cells was also demonstrated (Rajagopal and Kreitman 2000). Competition experiments with a large excess of antigen-purified LRP1 polyclonal antibodies indicated that cytotoxicity of both saporin and an ATF-saporin fusion could be competed in U937 cells (Fabbrini et al. 1997b). Mouse embryonic fibroblasts (MEF-2) derived from LRP1 knock-out mice were at least 10-fold less sensitive to saporin compared to MEF1 cells carrying both LRP1 and low-density lipoprotein receptor (LDLR) (Vago et al. 2005), indicating a role for LRP1 and possibly LDLR in saporin internalization in mouse fibroblasts. LB6 fibroblasts transfected with the human receptor for urokinase have been used to study internalization of a urokinase–saporin conjugate, again demonstrating a clear role for LRP1 in mediating saporin–conjugate internalization (Ippoliti et al. 2000).

While LRP1 is clearly involved in saporin uptake, at least in some cell lines, it does not appear to be essential for saporin cytotoxicity in other cases. Indeed, one study found no changes in sensitivity toward saporin between a control cell line and a CHO cell line down-regulated for LRP (Bagga et al. 2003a). This study made use of a mutant CHO cell line (CHO 13-5-1) that has no detectable LRP mRNA or protein, and exhibits a 100-fold increase in resistance to *Pseudomonas* exotoxin (PE) (Fitzgerald et al. 1995).

Saponins are low molecular weight compounds mainly produced by plants, including *S. officinalis*. They affect the plasma membrane of living cells and artificial membranes by interacting with cholesterol. Soapwort saponins have been shown to greatly enhance saporin cytotoxicity toward several different cell lines (Weng et al. 2008; Fuchs et al. 2009). This saponin-mediated cytotoxicity was affected by drugs interfering with clathrin-mediated endocytosis, while inhibitors of caveolae-mediated endocytosis had no influence (Weng et al. 2008). In both Vero and HeLa cells, chloroquine and bafilomycin A1 had no effect on saporin toxicity, indicating that saporin translocation to the cytosol is not dependent on the low pH of endosomal compartments (Vago et al. 2005). However, in the presence of saponins, saporin-mediated cytotoxicity in ECV-304 cells could be blocked by bafilomycin A1 (Weng et al. 2008). These results suggest that saporin may follow different intoxication pathways in the absence and in the presence of saponins.

The intracellular site from which saporin can escape to the cytosol remains unknown. However, several lines of evidence indicate that Golgi-mediated retrograde transport to the ER is not a prerequisite for saporin cytotoxic effect. While treatment with brefeldin A, a fungal metabolite that causes disassembly of the Golgi complex, blocks ricin and RTA cytotoxicity (Yoshida et al. 1991; Simpson et al. 1996), such an addition to saporin-treated cells fails to reduce toxicity (Vago et al. 2005). In addition, while appending the ER retrieval sequence KDEL has been shown to increase RTA cytotoxicity, presumably by promoting its retrograde transport to the ER (Wales et al. 1993), it does not enhance saporin cytotoxicity (Vago et al. 2005). Although the possibility that saporin reaches the ER via a Golgi-independent route cannot be excluded at this stage, the lack of any effect of brefeldin A and KDEL addition suggests that saporin may escape to the cytosol from a different compartment. In contrast to RTA, it seems unlikely that saporin takes advantage of the ERAD machinery to retrotranslocate to the cytosol because it does not reach the ER. ERAD mutants of Chinese hamster ovary (CHO) cells that were resistant to ricin and PE remained as sensitive to saporin as parental CHO cells (Teter and Holmes 2002; Geden et al. 2007). In addition, some other critical features of RTA are not shared with saporin. Lipid partitioning studies using Triton X-114 have demonstrated that while RTA is predominantly found in the detergent phase, the ricin B chain, ricin holotoxin and several Type I RIPs, including saporin, are instead found in the aqueous phase (Day et al. 2002). Importantly, RTA has been shown to interact with negatively charged lipid vesicles and with ER membranes, undergoing a conformational change that could make it a better substrate for the ERAD system (Mayerhofer et al. 2009). After dislocation to the cytosol, the low lysine content of RTA allows the toxin to avoid proteasomal degradation, most likely by hampering efficient ubiquitination (Deeks et al. 2002). Conversely, saporin is a very stable protein, does not stably associate with negatively charged vesicles, and is very rich in lysine residues (Santanché et al. 1997; Deeks et al. 2002; Fabbrini et al. 2003; Mayerhofer et al. 2009). Taken together, these data suggest that saporin and RTA use different strategies to reach the cytosol of mammalian cells.

Intracellular tracing of fluorescinated saporin in Vero cells and HeLa cells revealed the presence of saporin in punctuate structures. While no colocalization with early endosome and Golgi complex markers could be observed, the distribution of internalized saporin partially overlapped with that of Lamp1, a late endosome marker (Vago et al. 2005). Consistent with a role of endosome-located saporin in the intoxication process, endosomal membranes could be permeabilized using lypopolyamines or DMSO, thus increasing the access of saporin, but not of RTA, to the cytosolic compartment. In addition, two mutant CHO cell lines defective in endosomal to lysosomal transport were greatly sensitized to saporin (Geden et al. 2007). If saporin can reach the cytosol from an endosomal compartment, the translocation mechanism is not pH dependent since as mentioned above, toxicity is not affected by chloroquine or bafilomicyn A1 (Vago et al. 2005). The productive delivery route of endocytosed Type I RIPs in mammalian cells is still under investigation, and it would seem that these proteins can reach the

cytosolic compartment following as yet unidentified pathway(s). Dissecting the intracellular pathways leading to saporin cytosolic delivery will be particularly important in view of the therapeutic uses of chimeric molecules based on this plant toxin (Fabbrini et al. 2003).

The intoxication pathway followed by TC (a toxin used as an abortifacient that behaves as an invasive toxin, targeting syncythiotrophoblasts, macrophages, and T-cells) has been recently investigated (Zhang et al. 2009). TC binds cell surface receptors belonging to the LDL-related receptor family, and it has been suggested that the known abortifacient and renotoxic actions of TC are caused by LRP1-mediated uptake in trophoblasts and by LRP2/megalin-mediated uptake in proximal tubule epithelial cells (Chan et al. 2000). In agreement with this observation, Jurkatt-T cells (which are devoid of proteins belonging to the LDL receptor family) are essentially resistant to free TC, while in at least two target cell lines, JAR and K562, endocytosed TC was incorporated into "pomegrenade" vesicles deriving from multivesicular body (MVB) membranes, and was then secreted in association with these vesicles upon fusion of the MVB with the plasma membrane. These TC-loaded vesicles could mediate intercellular secretion, targeting both syngeneic and allogeneic cells, and were much more effective in delivering TC than when free toxin was administered. Whether other Type I RIPs, like saporin, are able to hijack this exosome-mediated intercellular traffic remains to be clarified.

5 Heterologous Expression of Saporin and Saporin Fusion Toxins

Initial attempts to express recombinant Type I RIPs in *E. coli* were problematic, as reported in the case of *Mirabilis* antiviral protein (Habuka et al. 1989), PAP (Chaddock et al. 1994), and saporin (Barthelemy et al. 1993). Both in vitro depurination assays (Hartley et al. 1991), and the finding that *E. coli* ribosomes are depurinated in vivo (Chaddock et al. 1994), confirmed that host toxicity was due to RIP catalytic activity. Still, recombinant saporin can be purified in active form directly from bacterial lysates or recovered after refolding from inclusion bodies, indicating that the toxic effect is not sufficient to completely compromise biosynthetic activity before a substantial amount of the toxin has been accumulated (Fabbrini et al. 1997a; Bagga et al. 2003a, b; Ghosh and Batra 2006; Pittaluga et al. 2005).

The BL21(λDE3)pLysS strain has become a popular bacterial host for RIP expression driven by T7 RNA polymerase, and has been used to produce recombinant PAP (Chaddock et al. 1994), dianthin (Legname et al. 1995), and saporin (or saporin fusions) (Lappi et al. 1994; Fabbrini et al. 1997a, b; Heisler et al. 2003; Giansanti et al. 2010). Expression in the absence of an inducer is very low due to the presence of T7 lysozyme, which represses transcription by T7 RNA polymerase. Indeed, using this bacterial host, no saporin was detectable in the absence of the

inducer, while a few mg of soluble protein per liter could be purified from bacterial lysates (Fabbrini et al. 1997a). Tight control of RIP expression may be crucial to obtain the recombinant protein in good yields. In fact, when saporin was expressed in the BL21(λDE3) strain (i.e., in the absence of T7 lysozyme), *E. coli* growth was clearly reduced even before induction of RIP expression. This toxicity was due to saporin catalytic activity, since it was not observed when a catalytic site mutant was expressed in the same host (Pittaluga et al. 2005).

Recently, a variant recombinant saporin carrying a C-terminal VSAV tetrapeptide (SapVSAV), recognized by a specific PDZ domain, has been expressed in the BL21(λDE3) host. The yield of soluble saporin improved when the corresponding PDZ domain was coexpressed. In this case, SapVSAV was most likely stabilized by the coexpressed PDZ domain (Giansanti et al. 2010).

Endotoxin contamination can be a disadvantage of bacterial expression systems (Fuchs et al. 2007). In addition, the expression of saporin fusions can be hampered by the inefficient folding of certain secretory domains in the bacterial cytosol. Therefore the development of robust eukaryotic expression systems for RIP-based immunotoxin expression would be highly desirable.

The ATF-saporin fusion provides an example of a chimeric toxin that is difficult to express at high levels and in soluble form in bacterial hosts. The urokinase system is involved in the metastatic spread of several tumors, and saporin fusions to the amino-terminal fragment of human urokinase (ATF) have several potential therapeutical applications. While only minute amounts of correctly folded fusion protein were recovered in soluble form when ATF-saporin was expressed in *E. coli* (Fabbrini et al. 1997b), the fusion protein was instead efficiently secreted in active form when targeted to the ER of *Xenopus laevis* oocytes (Fabbrini et al. 2000). However, preATF-saporin expression was found to be toxic to the oocyte, and injection of antisaporin neutralizing antibodies into the oocyte cytosol was required to eliminate host toxicity and increase the expression level (Fabbrini et al. 2000). The identification of a neutralizing antisaporin monoclonal antibody (or antibody fragment) to be used in intracellular immunization strategies may therefore be useful to counteract any toxic effect associated with saporin expression in eukaryotic cells (Fabbrini et al. 2003).

Recently, the methylotrophic yeast *Pichia pastoris* has been shown to be a suitable platform for the expression of saporin fusions (Lombardi et al. 2010). Saporin was expressed as a secretory precursor carrying the preproalpha mating factor leader sequence. Importantly, codon-optimization was found to be essential to obtain clones expressing high levels of active saporin. Although some toxicity toward the host was evident, saporin was efficiently processed by the removal of the preprosequence and accumulated in the culture medium at ∼30 mg/L, thus representing a several-fold improvement with respect to what had previously been obtained with the bacterial expression system (Fabbrini et al. 1997a). Secretion efficiency was found to be lower when saporin fusion chimeras were expressed, a fraction of the synthesized chimeric toxin being retained in the cell and degraded in the vacuole. Despite this, a model saporin immunotoxin and an ATF-saporin chimera could be expressed at several mg per liter of culture in shake flasks,

indicating that *P. pastoris* can be exploited as an expression platform for the production of therapeutic saporin chimeras (Lombardi et al. 2010).

6 Conclusions and Perspectives

Amongst the entries found while searching for RIPs in the PubMed database, a significant proportion also include the keyword "saporin." This reflects both the widespread use of saporin as a tool for the production of targeted toxins, and the increasing interest in the biochemical and functional characterization of this plant toxin. While these studies have clarified several aspects of saporin structure, catalytic activity, and intoxication processes, the role of the different saporin isoforms *in planta* is still poorly understood. Further work on the regulation of saporin biosynthesis and on saporin subcellular localization under normal and stress conditions may provide some hints on the elusive biological role of this and other plant RIPs. In addition, the identification of the subcellular compartment from which saporin escapes to the cytosol, and characterization of the translocation pathway, will contribute to our understanding of the mechanism by which certain proteins avoid the strict subcellular compartmentalization that characterizes eukaryotic cells. Finally, elucidating the molecular events responsible for saporin-mediated host toxicity will be of great help in the production of toxic chimeras for therapeutical applications.

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