Characterization of a saporin isoform with lower ribosome-inhibiting activity

M. Serena FABBRINI*¶ Emilia RAPPOCCIOLO*, Daniela CARPANI*, Michela SOLINAS†, Barbara VALSASINA‡, Umberto BREME‡, Ugo CAVALLARO*, Anders NYKJAER§, Ermanna ROVIDA*, Giuseppe LEGNAME‖ and Marco R. SORIA*

*Department of Biological and Technological Research – Dibit, San Raffaele Scientific Institute, via Olgettina 58, 20132 Milano, †Istituto Biosintesi Vegetali del Consiglio Nazionale delle Ricerche, via Bassini, 15, 20133 Milano, ‡Biotechnology Department, Pharmacia & Upjohn, via Giovanni XXIII 20014 Nerviano, Italy, §Department of Medical Biochemistry, University of Aarhus, Ole Worms Allè, DK-800 Aarhus C, Denmark, and ‖Italfarmaco Research Center, via dei Lavoratori 54, 20092 Cinisello Balsamo, Milano, Italy

We have expressed in *Escherichia coli* five isoforms of saporin, a single-chain ribosome-inactivating protein (RIP). Translation inhibition activities of the purified recombinant polypeptides *in vitro* were compared with those of recombinant dianthin 30, a less potent and closely related RIP, and of ricin A chain. Dianthin 30, and a saporin isoform encoded by a cDNA from leaf tissue (SAP-C), both had about one order of magnitude lower activity in translation inhibition assays than all other isoforms of saporin tested. We recently demonstrated that saporin extracted from seeds of *Saponaria officinalis* binds to α2-macroglobulin receptor (α2MR; also termed low density lipoprotein-receptor-related-protein), indicating a general mechanism of interaction of plant RIPs with the α2MR system [Cavallaro, Nykjaer, Nielsen and Soria (1995) Eur. J. Biochem. **232**, 165–171]. Here we report that SAP-C bound to α2MR equally well as native saporin. However, the same isoform had about ten times lower cytotoxicity than the other saporin isoforms towards different cell lines. This indicates that the lower cell-killing ability of the SAP-C isoform is presumably due to its altered interaction with the protein synthesis machinery of target cells. Since saporin binding to the α2MR is competed by heparin, we also tested in cell-killing experiments Chinese hamster ovary cell lines defective for expression of either heparan sulphates or proteoglycans. No differences were observed in cytotoxicity using native saporin or the recombinant isoforms. Therefore saporin binding to the cell surface should not be mediated by interaction with proteoglycans, as is the case for other α2MR ligands.

INTRODUCTION

Plants synthesize toxic ribosome-inactivating proteins (RIPs), that are *N*-glycosidases (EC 3.2.2.22) recognizing a specific adenine (A4324 in the rat) located in a universally conserved stem–loop region of 28S ribosomal RNA. The most representative RIP is ricin, which in addition to the catalytic A-chain subunit contains a B-chain that allows it to attach to and enter the cells. Conversely, single-chain or type I RIPs, like saporin, lack the B-chain [1]. RIPs might have great therapeutic potential as chimaeric toxins, obtained with either genetic [2] or biochemical manipulations, such as immunotoxins [3] or toxin conjugates [4,5]. Thus they are useful for treating cancer and autoimmune diseases and also against HIV infection [6]. By virtue of their antiviral properties, type I RIPs might also be used to improve defence mechanisms of transgenic plants of interest [7,8].

Saporin extracted and purified from seeds of *Saponaria officinalis* (SAP-S) was found initially to be heterogeneous at two amino acid positions, i.e. residues 48 (Asp or Glu) and 91 (Arg or Lys) ([9,10]; G. P. Nitti, unpublished work). This predicted the existence of at least four seed isoforms and, indeed, several different genomic clones were successively identified, confirming the existence of a multigene saporin family [11]. In addition, a leaf cDNA clone had been found to encode a saporin precursor, giving rise to a mature polypeptide of 253 amino acids that differed from SAP-S at 13 amino acid residues [12,13]. In contrast with type II RIPs, type I RIPs are active not only against eukaryotic but also against prokaryotic ribosomal RNA [14]. Initial attempts to express dianthin 30 [15], as well as other type I RIPs in *Escherichia coli*, did not involve tightly controlled systems of expression and were unsuccessful, as reported for Mirabilis antiviral protein [16], pokeweed antiviral protein (PAP) [17] and saporin [11]. Thus presumably type I RIPs are all toxic to *E. coli* ribosomes to various extents, whereas type II RIPs are not [14]. In addition, all RIPs display different specificities for ribosomes from different sources [1]. Indeed, RIPs must first interact with the complex structure of the ribosomes in order to recognize and then depurinate target rRNA. Therefore steps towards elucidating structure/function relationships among type I and type II RIPs would be highly desirable in order to engineer highly selective cytotoxins.

EXPERIMENTAL

Plasmids, strains and DNA manipulations

BL 21 (DE3) pLysS (Novagen) strain was used for expression of recombinant proteins. The pET-11d plasmid (Novagen) was used in all the constructs. A single *Nco*I site (CCATGA) provides the translational starting codon. A *Sac*II–*Eco*RI fragment from sequence 3 DNA [11] was ligated to pET-11d DNA digested with *Eco*RI and *Nco*I in the presence of a linker-adapter containing *Nco*I–*Sac*II sites. The pET-11d–SAP-3 construct was sub-

Abbreviations used: RIP, ribosome-inactivating protein; SAP, saporin isoform; α2MR, α2-macroglobulin receptor; PAP, pokeweed antiviral protein; RP-HPLC, reversed-phase HPLC; 3D, three-dimensional; TFA, trifluoroacetic acid; TCA, trichloroacetic acid; CHO, Chinese hamster ovary; RAP, receptor-associated protein; LpL, lipoprotein lipase; RNP, ribonucleoprotein.

¶ To whom correspondence should be addressed.

sequently engineered by substituting the original *Bam*HI–*Eco*RI fragment with 690 bp purified *Bam*HI–*Eco*RI genomic fragments from sequences 1, 4 and 6 genomic clones. This yielded pET-11d–SAP-l, pET-11d–SAP-4 and pET-11d–SAP-6 respectively. With sequence 6, an *Ssp*I restriction site allowed selection of recombinant clones. An *Nco*I restriction site could be used instead to select recombinants for the other saporin clones, since sequence 3 lacks this site. The saporin-coding leaf cDNA [12] was mutated to introduce a stop codon before the encoded C-terminal propeptide [13]. The resulting construct, pET-11d–SAP-C, was then fully sequenced to confirm that no changes were introduced during the amplification step. DNA sequencing was performed using the Pharmacia (Uppsala, Sweden) T7 sequencing kit. Oligonucleotides were synthesized with a 380B automatic DNA synthesizer (Applied Biosystems).

Expression and purification of recombinant saporin isoforms

Induction of expression of the toxic genes was essentially following manufacturer's instructions (Novagen). A single-step purification by ion-exchange chromatography was performed, loading soluble fractions of protein onto a Mono S® HR 5/5 FPLC® column as described [18]. Total *E. coli* extracts and fractions from column chromatography purifications were loaded onto 12.5 % and 15 % (w/v) polyacrylamide gels respectively. For Western blot analysis, proteins transferred onto nitrocellulose were probed with a rabbit anti-saporin antiserum at a 1:1000 dilution, and detected with goat anti-rabbit–horseradish peroxidase-conjugate antiserum [11]. Polyclonal rabbit antibodies directed against native saporin were generously given by D. A. Lappi, Advanced Targeting Systems, San Diego, CA, U.S.A.

Protein content in the peak fractions from ion-exchange chromatography purifications was determined with the Bio-Rad Protein Assay. Bovine serum albumin (Bio-Rad) and native saporin were utilized as standards.

Reversed-phase HPLC (RP-HPLC) and electrospray mass analysis

Native seed-extracted saporin, and all the recombinant isoforms were subjected to RP-HPLC on a Hewlett Packard 1090M apparatus (Wilmington, DE, U.S.A.) using a 1×250 mm, 218 TD C18 Vydac column (The Separation Group, Hesperia, CA, U.S.A.). Mobile phases A and B were respectively 0.1 % trifluoroacetic acid (TFA) in Milli-Q grade water and 0.078 % TFA in acetonitrile. Elutions were carried out with a linear gradient of buffer B from 25 % to 79 % in 25 min at a flow rate of 0.085 ml/min. Separations were performed at 50 °C and elution profiles were monitored with a Hewlett Packard 1040A Diode array detector at the wavelength of 215 nm.

On-line RP-HPLC/electrospray mass spectrometry was performed with samples of SAP-S, SAP-6 and SAP-C (a saporin isoform encoded by a cDNA from leaf tissue) on a Hewlett Packard 5989S MS-Engine single quadrupole instrument equipped with a Hewlett Packard 59987A electrospray interface. Eluates from the RP-HPLC were directly injected into the ion source of the mass spectrometer. The electrospray potential was approx. 6 kV. The quadrupole mass analyser was set to scan over a mass-to-charge ratio (m/z) from 1000 to 1700, at 2 s per scan for a total time of 10–12 s. The sum of data acquired over this time constituted the final spectrum. Molecular masses were calculated from several multiply-charged ions within coherent series. Mass calibrations were performed with horse skeletal muscle myoglobin (Sigma).

N-terminal sequence analysis

For protein sequence analysis, samples of SAP-3, SAP-4 and SAP-C after SDS/PAGE were electroblotted onto polyvinylidene difluoride membranes (Fluorotrans) at 300 mA for 45 min using 10 mM 3-(cyclohexylamino)-1-propanesulphonic acid (pH 11) in 10 % (v/v) methanol as transfer buffer. The membrane was stained for 1 min in Coomassie Blue R250/methanol/acetic acid (1:400:100, by vol.) and destained in 50 % methanol for 5 min. Bands were excised from the membrane and arranged in the cartridge block of the sequencer. Automated Edman degradation was performed on a pulsed-liquid-phase Sequencer, model 477 (Applied Biosystems) equipped with a Mod. 120 A HPLC instrument for detection of phenylthiohydantoin amino acids.

Biological assays of ribosome-inhibiting activities

Serial log dilutions ranging from 40 nM to 0.4 pM final concentration of each isoform in phosphate-buffered saline (PBS) were assayed in duplicate, dispensing 2 μl of each dilution in Eppendorf tubes on ice. A reaction mixture containing 2.5 μCi of tritiated leucine (L-[4,5-^{3}H]leucine, 45–85 Ci/mmol, Amersham International), 250 ng of brome mosaic virus RNA and 0.053 mM amino acid mixture without leucine was added in 3 μl samples. Nuclease-treated rabbit reticulocyte lysate (10 μl; Promega, Madison, WI, U.S.A.) thawed on ice was added to the assay tubes. Samples of 15 μl final volume were incubated at 30 °C for 60 min. The SAP-C dilutions tested range from 700 nM to 7 pM final concentration. At the end of incubations, samples were chilled on ice, brought to 0.1 mg/ml final concentration of ribonuclease A then further incubated at 23 °C for 20 min. To quantify the amount of radioactivity incorporated into translated protein, spots were made in triplicate on 3MM Whatman filter paper cut into small pieces. The filters were washed four times, 10 min each with 5 % ice-cold trichloroacetic acid (TCA; 5 ml/filter), then boiled for 1 min in 5 % TCA and washed with ice-cold 95 °C ethanol twice. Filters were dried at 65 °C for 30 min and then radioactivity was measured by liquid scintillation counting. Recombinant dianthin-30 mature polypeptide was also assayed for comparison, as well as recombinant ricin A chain (kindly supplied by J. Michael Lord, University of Warwick, U.K.). The concentration inhibiting translation by 50 % (IC_{50}) was 200 pM for recombinant ricin A chain when tested in these assays. The program MacALLFIT was used to process and evaluate the data from SAP-C, SAP-3 SAP-4 and SAP-S inhibition assays. Saporin RIP activities were also tested in rabbit reticulocyte lysates measuring inhibition of luciferase mRNA translation. Light emission of translated luciferase was measured in a Berthold LB Lumat luminometer. Inhibition of translation was reported as a decrease of light emission, i.e. as a percentage of control luciferase translated in the absence of saporin, which corresponds to 100 % of light emitted. Negative translation controls in the presence of the same concentrations of non-toxic proteins (carbonic anhydrase or bovine serum albumin) were identical with the control (results not shown). The IC_{50} of SAP-C was 125 pM whereas that of recombinant ricin A chain was 80 pM in these assays.

Cytotoxicity experiments

At least two independent cell-killing experiments were performed using each of the following cell lines: murine LB6, treated as described in [5], the human permanent cell line EA.hy 926, obtained by fusing human umbilical vein endothelial cells with

the tumour cell line A549 [19] and treated as in [20]. Three Chinese hamster ovary (CHO) cell lines: CHO-K1, the parental control cell line, and the two defective cell lines CHO-745 [21] proteoglycan-deficient, and CHO-677 [22] heparan sulphate-deficient, were kindly provided by J. Esko, University of California, La Jolla, CA, U.S.A. All three CHO cell lines were cultured in Ham's F12 medium (ICN, Costa Mesa, CA, U.S.A.) supplemented with 7.5% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin sulphate and plated at a density of 3.75×10^4 cells per ml in 80 μl/well. Briefly, cells were plated on gelatin-coated 96-well plates (Costar, Cambridge, MA, U.S.A.) 16–18 h before the experiments, and treated for either 24 h (LB6) or 48 h (CHO and EA.hy 926) in the absence or in the presence of serial log dilutions (ranging from 1 nM to 1000 nM final in 100 μl/ well) of either native saporin or the recombinant isoforms SAP-3 and SAP-C. Each point was tested in quadruplicate. At the end of the incubations in the presence of toxins, cells were washed with PBS then pulse-labelled for 2 to 4 h with L-[4,5-^{3}H]leucine (45–85 Ci/mmol, Amersham International) at 0.5 μCi/well. Total incorporation of radioactivity into protein was measured by harvesting cells on glass fibre filters and liquid scintillation counting. Cytotoxicity was calculated measuring the ID_{50}.

Binding of ^{125}I-labelled α2-macroglobin receptor (α2MR) to immobilized saporin

Wells of microtitre plates (Polysorp, Nunc, Denmark) were coated with 1 μg (100 μl of 10 μg/ml) of either native or recombinant saporin isoforms in 50 mM $NaHCO_3$, pH 9.6, for 2 h to provide about 80 ng (2.7 pmol) of immobilized saporin/well. After blocking with binding buffer (10 mM Hepes/140 mM NaCl/2 mM $CaCl_2$/1 mM $MgCl_2$, pH 7.8) containing 2% Tween-20 for 2 h at 20 °C, microtitre wells were washed three times and incubated with 5–10 pM ^{125}I-labelled α2MR in binding buffer containing 0.2% BSA for 16 h at 4 °C. Following a wash with binding buffer, bound radioactivity was eluted in 10% SDS and counted in a Packard (Meriden, CT, U.S.A.) gamma-counter. In the absence of immobilized saporin, binding of ^{125}I-labelled α2MR (blank value) amounted to less than 0.2%. All values have been corrected accordingly. For competition assays, ^{125}I-labelled α2MR (10 pM) was added to the wells in the presence of either 400 nM receptor-associated protein (α2MRAP) or 800 nM lipoprotein lipase (LpL, Sigma L-2254), which was dialysed overnight against binding buffer.

Multiple alignments, prediction of saporin secondary structure and three-dimensional (3D) structure comparisons

The sequences of saporin, trichosanthin, PAP, momorcharin and ricin used for our alignments were obtained from release 29 of the Swissprot sequence database. Multiple alignments were performed using the program PILEUP of the WISCONSIN package, version 8.0, based on the progressive alignment method [23] followed by manual adjustment. Co-ordinates of PAP (code lPAG), α-momorcharin (lAHB) and ricin A chain (lrtc) were obtained from the Brookhaven structure data bank [24]. The superposition of the known 3D structures was performed within the QUANTA (Molecular Simulations) molecular modelling package using a least-squares fitting algorithm [25]. For the secondary structure prediction of saporin, the method PHD [26] was used. The overall three-state accuracy was improved by up to 72% [26].

RESULTS

Construction and selection of clones coding for mature saporin isoforms

Figure 1 is a schematic representation of the strategy used to subclone the sequences coding for the mature polypeptides of the different saporin isoforms in the pET-11d expression vector. The isoform encoded by the sequence 3 genomic clone [11], coding for the isoform termed SAP-3, was previously expressed in *E. coli* fused to a bacterial signal sequence. Almost all the recombinant saporin was expressed intracellularly in an insoluble form, although some was exported to the periplasmic space. N-terminal sequencing of purified osmotic shock extracts indicated that part of the plant-encoded signal peptide was still present in the recombinant product. Yet the RIP activity of the recombinant saporin was 20 pM, almost identical with that of SAP-S [11]. Therefore the DNA of the genomic clone sequence 3 was digested with *Sac*II and *Eco*RI to remove the encoded leader peptide of saporin. Purified DNA was ligated to *Nco*I/*Eco*RI-cut pET-11d DNA in the presence of a specific adapter bearing *Nco*I and *Sac*II sites and restoring the missing coding sequence of mature SAP-3. Since all the differences in amino acid residues present

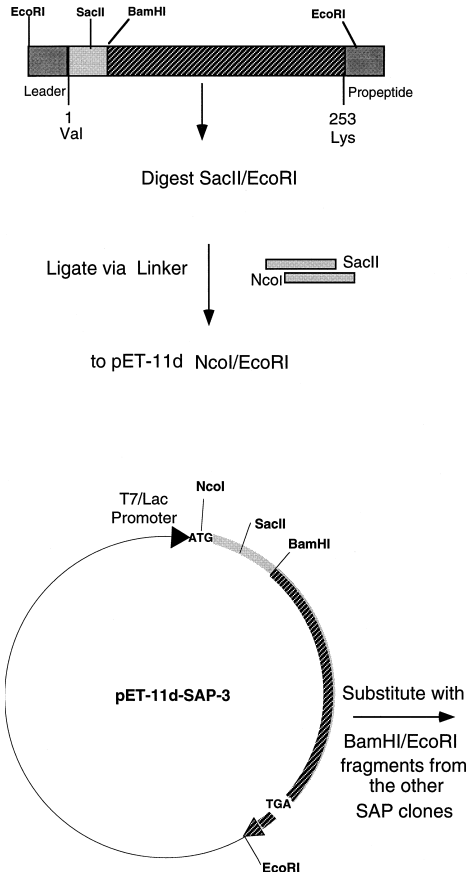

Figure 1 Schematic representation of the saporin-coding region and details of the pET-11d expression constructs

The N-terminal leader sequence and the C-terminal propeptide of preprosaporin (upper part of the Figure) are shown together with the first and the last amino acid residue found in mature saporin. The light grey box corresponds to the common N-terminal part of mature saporin. Restriction enzyme sites used for cloning the mature saporin-coding portions are also indicated.

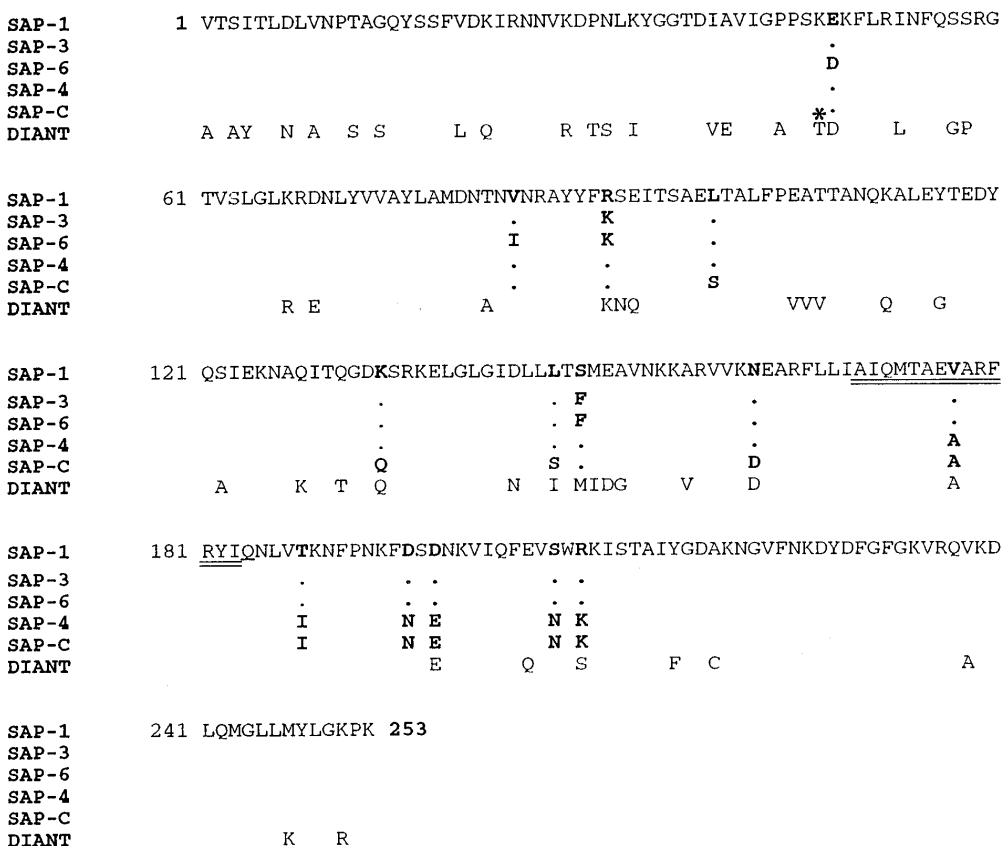

Figure 2 Protein sequence alignment of the saporin isoforms expressed in *E. coli*

The complete amino acid sequence of SAP-1 is shown. Dots identify conserved residues among SAP-1 and the other saporin isoforms, whereas variations in amino acid residues are indicated with bold type. Underlined is the ribosomal inactivating active site signature. The dianthin 30 (DIANT) sequence is also compared and aligned, and only the residues differing from SAP-1 are shown. The asterisk indicates that after Thr^{47} a second threonine is found in dianthin 30 which has been omitted to optimize the alignment.

among the other encoded isoforms are contained within a DNA stretch between two unique restriction sites, *Bam*HI and *Eco*RI, pET-11d–SAP-3 was used to obtain all subsequent constructs, substituting the original *Bam*HI–*Eco*RI fragment with those encoding the other isoforms (for details, see Experimental section). Thus only the ATG start codon is present before the sequence coding for the various mature saporin isoforms.

Expression and purification of saporin isoforms

In Figure 2 the aligned amino acid sequences of the recombinant mature saporins are shown. We have expressed the mature saporin polypeptides, termed SAP-1 and SAP-3 following the numbering of the respective genomic coding sequences [11], as well as the isoform termed SAP-6, as three representatives of seed-type isoforms. SAP-1 has one of the four possible seed-type amino acid patterns at residues 48 and 91; however, instead of having Phe^{149}, which is present in the two other seed-type isoforms, it has Ser^{149}, like the SAP-C isoform (Figure 2). In addition, SAP-6 has Ile^{84}, instead of Val, which is present in all the other isoforms; however, we cannot exclude the possibility that this variant might be an artifact due to the DNA amplification step. We also expressed a polypeptide closely resembling SAP-C, herein referred to as SAP-4, encoded by DNA sequence 4 [11]. Thus SAP-C and SAP-4 differ from all the other saporin isoforms only in a few residues, mainly located in a region of saporin close to the adenylate-binding site [11,27]. The protein sequence of dianthin 30 is also shown for comparison. When dianthin 30 was expressed in *E. coli* with the same host–vector system that we used here, the IC_{50} observed in cell-free inhibition assays was the same as that of native dianthin, i.e. about 300 pM [18]. SAP-C differs from SAP-4 in four residues (Figure 2), two of them being shared only with dianthin 30, i.e. Gln^{134} and Asp^{162}.

Tightly controlled conditions are required for efficient expression of the saporin and dianthin 30 genes in *E. coli*, since these recombinant RIPs are quite toxic to the bacterial host [14]. Non-induced or induced bacteria were lysed, sonicated and cell lysates were ultracentrifuged as described [18]; soluble and insoluble protein fractions were then analysed by SDS/PAGE, followed by immunoblot analysis using rabbit anti-saporin antiserum. No leaky expression of toxic saporin genes was observed before induction of T7 RNA polymerase. After induction, bacteria expressing the SAP-C isoform were growing at a faster rate than those expressing the other saporin isoforms, such that higher protein yields would be obtained both in the soluble and in the insoluble fractions. Yields of soluble recombinant seed-type saporin isoforms were between 1 and 3 mg/litre of culture, similar to those of recombinant PAP expressed using the same host–vector system described here [17]. However, yields were lower than those of the SAP-C isoform and of recombinant mature dianthin 30 (up to 10 mg/litre of culture

Table 1 HPLC-separated peak fractions of recombinant isoforms

Expected M_r values do not take into account the N-terminal methionine.

	Expected M_r	Mono-S® retention time (min)	RP-HPLC retention time (min)
SAP-1	28560	9.37	20.45
SAP-3	28592	9.92	21.54
SAP-6	28592	9.92	21.61
SAP-4	28556	8.63	19.83
SAP-C	28505	10.20	19.55

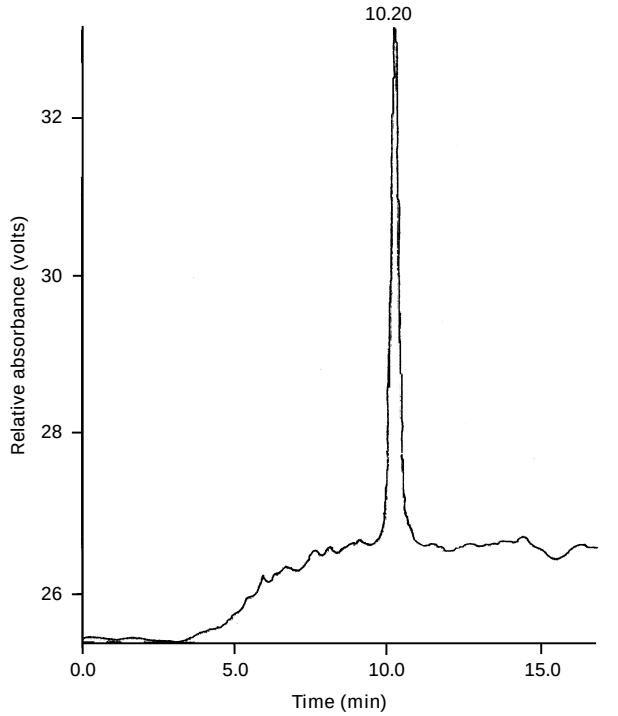

Figure 3 Ion-exchange chromatography purification of a saporin isoform

The elution profile of SAP-C is shown as representative of an HPLC purification performed after loading of *E. coli* soluble extracts: *x*-axis, time in min ($\times 10$); *y*-axis, UV monitoring expressed as differential of potential. Retention time (min) corresponds to the SAP-C elution peak.

[18]), suggesting that the latter RIPs possessed lower *E. coli* host toxicity (results not shown).

In all the extracts analysed, the soluble fraction contained the vast majority (90%) of recombinant protein, and was therefore used for purifying the different isoforms. A single-step purification was performed by ion-exchange HPLC, exploiting the high isoelectric point of saporin. All the isoforms eluted as single peaks around 150 mM NaCl, as did recombinant, mature dianthin 30 purified using the same procedure [18]. They also had similar retention times (Table 1), ranging from 8.63 min for SAP-4 to 10.20 min for SAP-C, whose elution profile is shown in Figure 3. The peak fractions from ion-exchange chromatography were analysed by SDS/PAGE followed by Coomassie Blue staining (Figure 4). The recombinant proteins had the expected relative molecular mass (M_r) of approx. 29000 as SAP-S. The three recombinant seed-type isoforms SAP-1, SAP-3 and SAP-6 behave

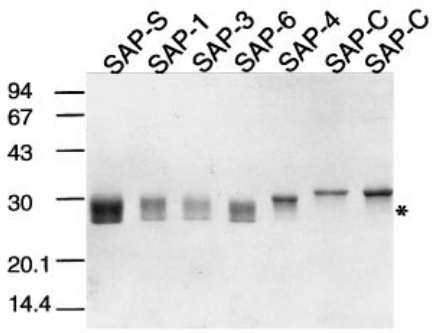

Figure 4 SDS/PAGE analysis of the recombinant, purified saporin isoforms

Equivalent amounts of the peak fractions (approx. 1.2 μg) from column chromatography purifications were run on a 15% polyacrylamide gel, then stained with Coomassie Brilliant Blue, with the exception of SAP-S and SAP-C (3 μg). The asterisk in the right-most lane identifies a faster migrating band in SAP-C. Markers ($10^{-3} \times M_r$) are also indicated.

similarly, showing electrophoretic patterns most resembling that of non-recombinant, native saporin extracted and purified from seeds (SAP-S). The presence of a diffuse band, almost appearing as two bands with a smear between them, was always observed with SAP-S, and was suggested to be an artifact due to the isoelectric point of 10.5 measured for native saporin [28]. Conversely, both SAP-C and SAP-4 isoforms migrated as a sharper band with slightly lower electrophoretic mobility (Figure 4). However, their theoretical pIs cannot account for their different mobilities. By increasing the amount of SAP-C loaded onto the gels, a faint faster-migrating band can also be detected (Figure 4, asterisk). Interestingly, it has been reported that leaf-extracted saporins show on SDS/PAGE an M_r higher than the seed-extracted protein by approx. 1500–2000 [29].

Biochemical characterization of recombinant saporin isoforms

To assess the purity of the peaks from our ion-exchange chromatography purifications, the recombinant isoforms were also subjected to RP-HPLC. A single peak was eluted in each case, as shown for SAP-C (Figure 5, top). Retention times obtained with each recombinant isoform are summarized in Table 1.

In addition, the M_r was accurately estimated by electrospray analysis of SAP-C and SAP-6 isoforms. For comparison, native seed-extracted saporin was also analysed. The mass spectrum of SAP-S was centred on the value of M_r 28560 when directly injected in the ionization chamber. However, since SAP-S is known to be heterogeneous, the mass spectrum analysis was performed with SAP-S previously subjected to RP-HPLC purification. Figure 5 (bottom) shows that three peaks were eluted by RP-HPLC, having retention times of 19.83, 20.44 and 21.65 min. The two main peaks accounted for about 35% and 60% of the total protein respectively. Mass spectra of peak A were resolved to three values of M_r 28546, 28555 and 28557. Peak B yielded a single value of M_r 28557. The same analysis was performed with the recombinant isoforms SAP-C and SAP-6, coupling RP-HPLC separation to electrospray mass analysis. The single RP-HPLC peak of SAP-C was resolved into two mass values of 28502.4 and 28617.6, representing respectively 46% and 54% of the total. A similar situation was observed with SAP-6. The RP-HPLC peak was resolved into two mass values of 28574 and 28715 representing respectively 24% and 76% of the total. Therefore, in the case of the recombinant isoforms, the

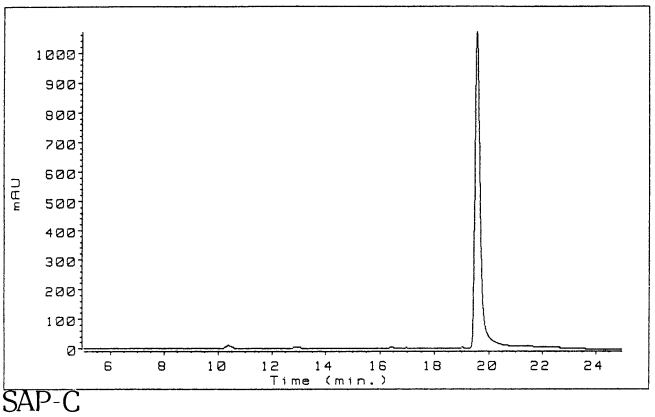

Figure 5 RP-HPLC analysis of SAP-S and SAP-C

(Top panel) The elution profile of SAP-C is shown as a representative example. All recombinant isoforms were loaded onto a C18 Vydac column and eluted using a linear gradient from 25 to 79% (v/v) acetonitrile at 50 °C. A single peak was obtained in each case. *y*-axis: UV monitoring expressed as differential of potential. (Bottom panel) For comparison, elution profile of native seed-extracted saporin.

Table 2 Comparison between SAP-3 and SAP-C RIP activities

*Means ± S.E.M. of at least two experiments each counted in triplicate. †Means ± S.D. of one representative experiment performed in quadruplicate. N.D. = not determined.

		SAP-3	SAP-C	SAP-S
Cell-free IC_{50}* (pM)		17 ± 4	157 ± 27	12 ± 5
Cell lines ID_{50}† (nM)	EA.hy 926	20 ± 5	200 ± 30	N.D.
	LB6	50 ± 15	500 ± 50	N.D.
	CHO-K1	150 ± 30	> 1000	90 ± 10
	CHO-745	150 ± 30	> 1000	100 ± 20
	CHO-677	150 ± 25	> 1000	150 ± 30

presence of two mass spectra should be solely related to either the presence or the absence of an N-terminal methionine. The accuracy of mass determinations was 99.9% (Table 1).

The RP-HPLC coupled to electrospray mass analysis of recombinant seed-type isoform SAP-1 (which correspond to the same batch of preparation of the protein loaded onto the SDS/PAGE) revealed the presence of a single mass of 28683, thus corresponding to 100% of polypeptide still bearing the initiator Met. The electrophoretic pattern shows, however, two migrating bands as observed with the native seed-extracted saporin.

Table 3 Binding of SAP-C to the α2MR

Binding of ^{125}I-labelled α2MR was measured as described in the Experimental section and is expressed as the percentage of added tracer. Data are means ± S.D. of a representative experiment performed in triplicate. N.D. = not determined.

	^{125}I-labelled α2MR bound (%)	
	SAP-C	SAP-S
Total binding	12.42 ± 1.8	11.89 ± 2.3
+ EDTA (5 mM)	3.87 ± 0.22	3.81 ± 0.62
+ heparin (10 IU/ml)	0.92 ± 0.34	1.68 ± 0.30
+ LpL (800 nM)	0.88 ± 0.11	N.D.
+ RAP (400 nM)	1.40 ± 0.53	N.D.

The isoforms SAP-3, SAP-4 and SAP-C were further characterized by N-terminal sequencing. After PVDF-blotting either the smeared band of SAP-3 or those corresponding to SAP-4 and SAP-C were excised and microsequenced. Each isoform had either Met or Val as first amino acid residue, as detected by automated Edman degradation of the purified polypeptides. The relative percentages of Met and Val were respectively 52% and 48% for SAP-3, and 60% and 40% for both SAP-4 and SAP-C.

Ribosome-inactivating activity of recombinant saporin isoforms

The specific ribosome-inhibiting capabilities of the natural and of the recombinant proteins were compared using a cell-free translation system (Experimental section), assaying activities of these RIPs in serial log dilutions. The saporin isoforms SAP-1, SAP-6, SAP-4 and SAP-3 all had IC_{50} values of approx. 10–20 pM, like that of SAP-S, as previously observed [11]. In contrast, SAP-C showed an IC_{50} of 175 pM in the range of activity of the less potent RIP dianthin 30. Processing these data using a statistical program, MacALLFIT, yielded the IC_{50}s for SAP-3, SAP-C and SAP-S shown in Table 2.

We next compared the cytotoxicity of the recombinant proteins in cell-killing experiments, testing LB6 murine cells and the human hybrid permanent cell line EA.hy 926. In two independent experiments, SAP-3 was equally cytotoxic with SAP-S on both LB6 [5] and EA.hy 926 [20] cells, whereas SAP-C had lower cytotoxicity (approx. 10-fold less than that of SAP-3) with both LB6 and EA.hy 926 (Table 2) cells.

Great efforts are currently ongoing to identify sequential events starting from cell-surface toxin binding to the final step, taking place in the cytosol, of ribosome depurination. Most of these studies have been carried out on the internalization pathway of the ricin holotoxin [30]. We previously demonstrated that saporin entry into the cells is not a passive mechanism as was generally believed, but is mediated by a large and widespread receptor, the α2MR [31]. α2MR is a multifunctional endocytic receptor bearing multiple binding sites [32]. Therefore we performed solid-phase binding experiments to α2MR, comparing SAP-S with the recombinant SAP-3 and SAP-C isoforms. In this assay, SAP-3 was able to bind to α2MR as efficiently as SAP-S (A. Nykiaer, unpublished work). In addition, SAP-C also bound equally well to α2MR (Table 3). Binding could be inhibited by EDTA, heparin and both the competitors LpL and RAP, as

previously shown for SAP-S [31]. This confirms the specificity of α2MR binding (Table 3).

Finally, since heparin competed with the binding of saporin isoforms to α2MR, we compared the cytotoxicities of SAP-S, SAP-3 and SAP-C in CHO cell lines defective in proteoglycan biosynthesis [22]. We tested mutant 745, which lacks one of the first acting enzymes, xylosyltransferase; therefore, assembly of both heparan sulphate and chondroitin sulphate does not take place. This mutant has less than 15% proteoglycans compared with wild-type CHO cells. Conversely, mutant 677 is defective specifically in heparan sulphate biosynthesis but makes about three times as much chondroitin sulphate as the wild-type cell line. When we tested either SAP-S or the recombinant isoforms on these CHO cell lines, no differences could be observed in specific cytotoxicity between wild-type and mutant CHO cells (Table 2).

DISCUSSION

Plants express several RIP isoforms in a tissue-dependent [33,34] and season-dependent fashion [1,35], but the reason for such wide heterogeneity remains unclear. This might represent a plant defence mechanism. Indeed, depurination of tobacco ribosomes catalysed by several RIPs correlates, for instance, with their antiviral activity in the infected plants [8]. Moreover, ribosomes from several species of dicotyledonous plants are sensitive to their own RIP, including PAP [36], dianthin 32 [37] and saporin [2].

In this study we have expressed several saporin isoforms in *E. coli* and compared their RIP activities, as a first step to determining whether amino acid variations present among them could reflect different specificities or catalytic properties. Three seed-type (SAP-1, SAP-3, SAP-6) as well as two closely related isoforms termed SAP-C and SAP-4 were selected for expression.

After purification of the recombinant isoforms, we observed that seed-type isoforms migrated on SDS/PAGE with a pattern similar to that of native saporin, whereas SAP-C and SAP-4 showed a different electrophoretic behaviour. However, by RP-HPLC only one single peak was eluted on loading each of the recombinant isoforms, while SAP-S was confirmed to be heterogeneous. Although the electrophoretic mobility of SAP-C is altered, accurate mass analysis showed that SAP-C has an M_r consistent with its theoretical one. Therefore the reason for the heterogeneity in the electrophoretic mobilities of our saporin isoforms remains unclear. Similarly aberrant electrophoretic mobilities had been observed previously, e.g. a decrease in mobility after removal of a protein's signal peptide [38]. Also, the relative mobility of a mutant G-protein was affected by a single amino acid change [39].

The specific inhibitory activity of the saporin isoforms was compared with that of seed-extracted saporin and of recombinant dianthin 30. Only the recombinant highly expressed leaf isoform, SAP-C, showed similar potency to dianthin 30, whereas all the other recombinant isoforms had similar RIP activity to the seed-extracted saporin. Saporin isoforms extracted from leaves of *S. officinalis* showed almost one order of magnitude lower inhibitory activity in rabbit reticulocyte lysates, when compared with seed isoforms extracted from the same plants [34]. However, only the N-terminal sequences of these isoforms were determined and none of them matches the recombinant isoforms expressed in our work [34].

A truncated saporin lacking the first 28 residues of the mature peptide had virtually no depurinating activity [40]. Indeed, Tyr^{16} and Arg^{24} are invariant residues, present in saporin and dianthin as well, which are thought to play a crucial role in stabilizing a helix bend, allowing the two catalytic residues Glu^{177} and Arg^{180} of ricin to interact [41]. Also, it was demonstrated that aromatic and charged residues in the first α-helix of ricin A chain, at the N-terminus, are necessary for ricin activity [42]. Iodination of extractive saporin at Tyr residues results in protein aggregation and, presumably, in an inactive RIP (U. Cavallaro, unpublished work). Thus arginine and tyrosine residues at the N-terminus might be involved in correct saporin folding as well. In this work, N-terminal sequencing of recombinant SAP-C confirmed that no degradation at the N-terminus was present. The presence of the initiator methionine should not affect the catalytic RIP activity of either recombinant saporin isoform and might simply reflect a low efficiency in its removal by the *E. coli* methionyl-amino-peptidase. Mis-folding of the SAP-C isoform was ruled out because SAP-C was expressed at high level in soluble form and was resistant to protease degradation to the same extent as SAP-S (M. S. Fabbrini, unpublished work). Far-UV circular dichroism (CD) analysis showed that the CD spectra of SAP-4 and SAP-C were almost indistinguishable and accounted for an $\alpha+\beta$-type architecture, as found in ricin A chain (G. Fossati, unpublished work).

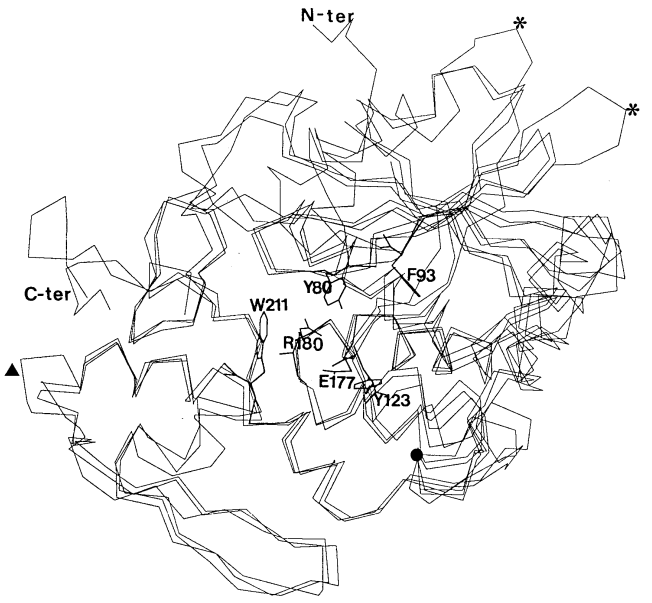

Figure 6 Superposition of Cα traces

Cα traces of superposed X-ray structures of ricin A chain, PAP and momorcharin are shown. The N-terminus and the prominent loop at the C-terminus of ricin A chain are indicated together with the position of key active site residues. The asterisks identify the loops (80–85) and (107–111) protruding from the PAP structure, and the solid triangle corresponds to a loop (203–206) in momorcharin. The solid circle marks the putative position of the saporin exposed residue Lys^{134}, which is substituted by Gln in SAP-C. ter, terminus.

Ricin A-chain and crystallized type 1 RIPs share the same overall tri-dimensional folding pattern, despite sharing only about 30% sequence similarity [43]. However, all residues in the catalytic active-site cleft of ricin A chain are conserved among type 1 and type 2 RIPs (Figure 6). Therefore mutation of these same key catalytic residues in other RIPs always results in a drastic loss of activity, as recently demonstrated for the type 2 RIP abrin [44].

To identify regions involved in substrate recognition and binding, we aligned several RIP sequences with their corresponding X-ray-structure-derived secondary structures. A pre-

diction of the secondary structure of seed-type saporin was obtained by multiple alignment using the neural network system PHD. Structurally conserved regions among the RIPs correspond to regions of high local amino acid similarity. However, major structural differences between PAP and ricin A-chain do not seem to account for their differing ribosome specificity [45]. Molecular electrostatic potential distribution, calculated for residues close to the adenylate-binding site, mapped onto the solvent surface, indicating that there is considerable variation between ricin, PAP and dianthin 30. This could account for the differences in ribosome specificity exhibited by these RIPs [46]. Thus exposed residues at putative RNA-binding domains might be likely candidates responsible for the heterogeneity observed between RIP activities.

Proteins binding RNA contain one or more copies of a putative RNA-binding domain consisting of two ribonucleoprotein (RNP) consensus motifs: a hydrophobic hexapeptide stretch, RNP-2, and an octapeptide motif, RNP-1 [47]. A RNP-like structural motif was identified in ricin A chain [42] that shows similarity to the recently solved 3D structure of the RNP motif of UlA spliceosomal protein [48]. This motif overlaps with a RNP domain tentatively identified in the RIPs we examined, including a putative hydrophobic RNP-2 found in the core β-sheet that contains the active-site residue Tyr^{80} involved in sandwiching together with Tyr^{123} the formycin monophosphate analogue at the adenylate-binding site. Within this RNP-2 like motif, the first three positions are conserved among saporin and other RNA-binding proteins [49]. Conserved residues in the RNP-1- and RNP-2-like motifs might be critical for the RIP's association with rRNA, whereas exposed residues in the most variable regions, especially in unstructured loops, may account for differences in specificity. From our putative model of saporin structure, we predict that Lys^{134} of the saporin sequence (which is substituted by Gln^{134} in SAP-C and dianthin-30) is located at a conserved surface loop found in the putative RNA-binding domain (Figure 6). Therefore we postulate that the difference in SAP-C activity might be due to impaired RIP–ribosome interaction.

To test this hypothesis, SAP-C was assayed in cytotoxic experiments. If steps from receptor binding and internalization to retrograde transport along the endomembrane system, to toxin translocation to the cytoplasm, were as efficient for SAP-C as for native saporin and only ribosome recognition was impaired, we would expect to observe a similar difference in potency between SAP-C and the seed-type isoforms, as found in the cell-free inhibition assays. Indeed, SAP-C was about ten-fold less cytotoxic irrespective of the cell line tested. Since SAP-C was able to bind efficiently to the putative receptor mediating saporin internalization, our data clearly support this hypothesis. Conversely, the polymorphism of saporin seed-type isoforms, involving residues at positions 48 and 91 that are also located in loop regions, presumably did not affect substrate recognition and activity, at least in reticulocyte lysates. The three other SAP-C substitutions compared with SAP-4 are found in α-helix structures. Although we cannot exclude the possibility that they contributed to the observed lower ribosome-inhibiting activity, these residues are presumably not accessible to the solvent surface.

Finally, to further investigate the role of proteoglycans in saporin internalization, we tested mutant CHO cell lines in cell-killing experiments. The same extent of loss of potency was obtained again when comparing the cytotoxicity of SAP-C with that of SAP-3 and SAP-S. However, since no significant differences in cytotoxicity were observed among seed-type saporin isoforms against the mutant CHO cells, heparan sulphate proteoglycans do not seem to facilitate the concentration of saporins at the cell surface, as was shown for other α2MR ligands [50,51].

This work is dedicated to the memory of Gianpaolo Nitti. We are grateful to Gaetano Orsini, Lucia Monaco, Vinod Singh and Douglas A. Lappi for helpful discussions and to Aldo Ceriotti and Luca Benatti for critical reading of the manuscript. We also thank Jan Malyszko for oligonucleotide synthesis and Gianluca Fossati for the circular dichroism spectroscopy data. This research was supported by Consiglio Nazionale delle Ricerche PF ACRO, Rome, the Associazione Italiana per la Ricerca sul Cancro, Milan, the Italian National AIDS Research Project, and Regione Lombardia.

REFERENCES

1 Stirpe, F., Barbieri, L., Battelli, M. G., Soria, M. R. and Lappi, D. A. (1992) Bio/Technology **10**, 405–412
2 Lappi, D. A., Ying, W., Barthelemy, I., Martineau, D., Prieto, I., Benatti, L., Soria, M. and Baird, A. (1994) J. Biol. Chem. **269**, 12552–12558
3 Falini, B., Bolognesi, A., Flenghi, L., Tazzari, P. L., Broe, M. K., Stein, H., Durkop, H., Aversa, F., Corneli, P., Pizzolo, G., Barbabietola, G., Sabattini, E., Pileri, S., Martelli, M. F. and Stirpe, F. (1992) Lancet **339**, 1195–1196
4 Casscells, W., Lappi, D. A., Olwin, B. B., Wai, C., Siegman, M., Speir, E. H., Sasse, J. and Baird, A. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 7159–7163
5 Cavallaro, U., del Vecchio, A., Lappi, D. A. and Soria, M. R. (1993) J. Biol. Chem. **268**, 23186–23190
6 McGrath, M. S., Hwang, K. M., Caldwell, S. E., Gaston, I., Luk, K., Wu, P., Ng, V. L., Crowe, S., Daniels, J., Marsh, J., Deinhart, T., Lekas, P. V., Vennari, J. C., Yeung, H. W. and Lifson, J. D. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2844–2848
7 Lodge, J. K., Kaniewski, W. K. and Tumer, N. E. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7089–7093
8 Taylor, S., Massiah, A., Lomonossoff, G., Roberts, L. M., Lord, J. M. and Hartley, M. (1994) Plant J. **5**, 827–835
9 Maras, B., Ippoliti, R., De Luca, E., Lendaro, E., Bellelli, A., Barra, D., Bossa, F. and Brunori, M. (1990) Biochem. Int. **21**, 631–638
10 Barra, D., Maras, B., Schinina, E., Angelaccio, S. and Bossa, F. (1991) Biotechnol. Appl. Biochem. **13**, 48–53
11 Barthelemy, I., Martineau, D., Ong, M., Matsunami, R., Ling, N., Benatti, L., Cavallaro, U., Soria, M. and Lappi, D. A. (1993) J. Biol. Chem. **268**, 6541–6548
12 Benatti, L., Saccardo, M. B., Dani, M., Nitti, G. P., Sassano, M., Lorenzetti, R., Lappi, D. A. and Soria, M. (1989) Eur. J. Biochem. **183**, 465–470
13 Benatti, L., Nitti, G., Solinas, M., Valsasina, B., Vitale, A., Ceriotti, A. and Soria, M. R. (1991) FEBS Lett. **291**, 285–288
14 Hartley, M. R., Legname, G., Osborn, R., Chen, Z. and Lord, J. M. (1991) FEBS Lett. **290**, 65–68
15 Legname, G., Gromo, G., Lord, J. M., Monzini, N. and Modena, D. (1993) Biochem. Biophys. Res. Commun. **192**, 1230–1237
16 Habuka, N., Murakami, Y., Noma, M., Kudo, T. and Horikoshi, K. (1989) J. Biol. Chem. **264**, 6629–6637
17 Chaddock, J. C., Lord, J. M., Hartley, M. R. and Roberts, L. M. (1994) Nucleic Acids Res. **22**, 1536–1540
18 Legname, G., Fossati, G., Monzini, N., Gromo, G., Marcucci, F., Mascagni, P. and Modena, D. (1995) Biomed. Pept. Proteins Nucleic Acids **1**, 61–68
19 Edgell, C. J., McDonald, C. C. and Graham, J. B. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3734–3737
20 Cavallaro, U., del Vecchio, A., Tazzari, P. L., Massazza, G. and Soria, M. R. (1993) Drug Delivery **1**, 119–124
21 Esko, J. D., Stewart, T. E. and Taylor, W. H. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 3197–3201
22 Esko, J. D., Rostand, K. S. and Weinke, J. L. (1988) Science **241**, 1092–1096
23 Feng, D. F. and Doolittle, R. F. (1987) J. Mol. Evol. **25**, 351–360
24 Bernstein, F. C., Koetzle, T. F., Williams, G., Meyer, E. F., Brice, M. D., Rodgers, J. R. and Kennard, O. (1977) J. Mol. Biol. **112**, 535–542
25 Sutcliff, M. J., Haneef, I., Carney, D. and Blundell, T. (1987) Protein Eng. **1**, 377–384
26 Rost, B. and Sanders, C. (1994) Proteins **123**, 123–125
27 Ready, M. P., Katzin, B. J. and Robertus, J. D. (1988) Proteins **3**, 53–59
28 Lappi, D. A., Esch, F., Barbieri, L., Stirpe, F. and Soria, M. (1985) Biochem. Biophys. Res. Commun. **129**, 934–942
29 Carzaniga, R., Sinclair, L., Fordham-Skelton, A. P., Harris, N. and Croy, R. R. D. (1994) Planta **194**, 461–470
30 Lord, J. M., Roberts, L. M. and Robertus, J. D. (1994) FASEB J. **8**, 201–208
31 Cavallaro, U., Nykjaer, A., Nielsen, M. and Soria, M. R. (1995) Eur. J. Biochem. **232**, 165–171
32 Moestrup, S. K. (1994) Biochim. Biophys. Acta **1197**, 197–213
33 Reisbig, R. R. and Bruland, O. (1983) Arch. Biochem. Biophys. **224**, 700–706

34 Ferreras, J. M., Barbieri, L., Girbes, T., Battelli, M. G., Rojo, M. A., Arias, F. J., Rocher, M. A., Soriano, F., Mendez, E. and Stirpe, F. (1993) Biochim. Biophys. Acta **1216**, 31–42
35 Houston, L. L., Ramakrishnan, S. and Hermodson, M. A. (1983) J. Biol. Chem. **258**, 9601–9604
36 Taylor, B. E. and Irvin, J. D. (1990) FEBS Lett. **273**, 144–146
37 Prestle, J., Shonfelder, M., Adam, G. and Mundry, K. W. (1992) Nucleic Acids Res. **20**, 3179–3182
38 Fabbrini, M. S., Valsasina, B., Nitti, G., Benatti, L. and Vitale, A. (1991) FEBS Lett. **286**, 91–94
39 Machamer, C. E. and Rose, J. K. (1988) J. Biol. Chem. **263**, 5948–5954
40 Fordham-Skelton, A. P., Taylor, P., Hartley, M. R. and Croy, R. (1991) Mol. Gen. Genet. **229**, 460–466
41 Montfort, W., Villafranca, J. E., Monzingo, A. F., Ernst, S. R., Katzin, B., Rutenber, E., Xuong, N. H., Hamlin, R. and Robertus, J. D. (1987) J. Biol. Chem. **262**, 5398–5403
42 Munishkin, A. and Wool, I. G. (1995) J. Biol. Chem. **270**, 30581–30587
43 Monzingo, A. F., Collins, E. J., Ernst, S. R., Irvin, J. D. and Robertus, J. D. (1993) J. Mol. Biol. **233**, 705–715
44 Hung, C. H., Lee, M. C., Chen, J. K. and Lin, J. Y. (1994) Eur. J. Biochem. **219**, 83–87
45 Chaddock, J. A., Monzingo, A. F., Robertus, J. D., Lord, J. M. and Roberts, L. M. (1996) Eur. J. Biochem. **235**, 159–166
46 Bravi, G., Legname, G. and Chan, A. W. (1995) J. Mol. Graphics **13**, 83–88
47 Bandziulis, R. J., Swanson, M. S. and Dreyfuss, G. (1989) Genes Dev. **3**, 431–437
48 Oubridge, C., Ito, N., Evans, P. R., Teo, C. H. and Nagai, K. (1994) Nature (London) **372**, 432–438
49 Query, C. C., Bentley, R. C. and Keene, J. D. (1989) Cell **57**, 89–101
50 Mikhailenko, I., Kounnas, M. Z. and Strickland, D. K. (1995) J. Biol. Chem. **270**, 9543–9549
51 Nykjaer, A., Nielsen, M., Lookene, A., Meyer, N., Roigaard, H., Etzerodt, M., Beisiegel, U., Olivecrona, G. and Gliemann, J. (1994) J. Biol. Chem. **269**, 31747–31755

Received 16 February 1996/22 October 1996; accepted 12 November 1996