Ebulin 1, a Nontoxic Novel Type 2 Ribosome-inactivating Protein from Sambucus ebulus L. Leaves*

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A novel type 2 ribosome-inactivating protein (RIP) that we named ebulin 1 has been isolated from leaves of Sambucus ebulus L. (Caprifoliaceae). In vitro ebulin 1 strongly inhibited protein synthesis by rabbit reticulocyte lysates, rat brain, and rat liver cell-free systems but did not affect in vitro plant nor bacterial protein synthesis. Ebulin 1 is composed of two subunits, a catalytic A subunit (M, 26,000) and a D-galactose-binding lectin B subunit (M, 30,000). Amino-terminal amino acid sequence homology revealed the novelty that the ebulin 1 A-chain shares a high degree of homology not only with the A-chain of other type 2 RIPs but rather with the Cucurbitaceae type 1 RIP bricodin S and the anti-human immunodeficiency virus type 1 proteins trichosanthin and TAP 29. Upon treatment with acid aniline the rRNA from ebulin 1-treated rabbit reticulocyte ribosomes released the RNA fragment which is diagnostic of RIP catalytic action. Ebulin 1 was nontoxic to mice up to 2 mg/kg of body weight and did not inhibit protein synthesis in cultured NHC human epithelial cells which are highly sensitive to ricin.

Ribosome-inactivating proteins (RIPs)* are plant toxins with N-glycosidase activity on the large rRNA of mammalian, fungal, plant, and bacterial ribosomes that irreversibly impair protein synthesis (1, 2). The action of RIPs on ribosomes abolishes ribosomal ability to interact with elongation factors 2 or G and thus irreversibly arrests polypeptide chain elongation (1). The molecular action of RIPs involves the depurination of the largest rRNA which upon treatment with acid aniline releases the diagnostic RNA fragment (2-4). The bases released by RIPs are A, C, G, and T of rat liver 28 S rRNA (2), A and T of yeast 26 S rRNA (3), and A and G of Escherichia coli 23 S rRNA (4). RIPs have been classified as type 1, those consisting of a unique enzymic polypeptide chain, and type 2, those consisting in one or two dimers of two different polypeptide chains linked by a disulfide bridge, one being the enzymic chain and the other a lectin able to recognize membrane sugars, mostly galactose residues (5, 6). Type 1 RIPs are relatively abundant and to date nearly 30 have been isolated (1). By contrast, type 2 RIPs are very rare, and since the isolation in 1888 by Stillmark of the first one, the toxic lectin ricin (1), until now only five have been detected and isolated (1, 6).

While the biological role of RIPs in plant producers is unknown, interest in these proteins is increasing due to their potential use as a toxic moiety in immunotoxins for the treatment of several important human diseases such as cancer (7-9) and AIDS (10, 11). Furthermore, some RIPs have antiviral activity against animal and plant viruses (12, 13). Very recently it has been shown that the type 1 RIP trichosanthin (14), pokeweed antiviral protein (11), MAP 30 (15), and TAP 29 (16) are agents that are directly active against anti-human immunodeficiency virus type 1. Additionally, trichosanthin has very recently been used in clinical trials for AIDS therapy (17, 18).

During our screening studies searching for new RIPs we found that Sambucus ebulus L. (belonging to Caprifoliaceae) had RIP activity. We have named the new RIP, ebulin 1, which strongly inhibited protein synthesis, displayed the rRNA N-glycosidase activity on mammalian ribosomes, which is characteristic of RIPs, and is not toxic to mice nor to cultured mammalian cells. This allowed us to classify ebulin 1 as a new type 2 ribosome-inactivating protein such as ricin, abrin, viscumain, modecinn, and volkensin (1) but of a new class, the nontoxic type 2 RIPs.

EXPERIMENTAL PROCEDURES

Materials—Current chemicals were of the highest purity available and most of them were from Merck (Darmstadt, Germany) and Boehringer Mannheim. Hemine, phenylhydrazine, amino acids, and the bark lectin from Sambucus nigra were from Sigma, Acrylamide, bis-acrylamide, and phenol were from Boehringer Mannheim. Ethidium bromide was from Fluka (Buchs, Switzerland). Ready-?e scintillation mixture was from Beckman Instruments. Poly(vinylidene) fluoride membranes were purchased from Millipore Iberica (Madrid, Spain); tP[N]-Methionine (Translabel); specific activity, 1164 Ci/m mol) was obtained from ICN through Nuclear Iberica (Madrid, Spain); L-5H]phenylalanine (specific activity, 111 Ci/mmol) and L-5H]valine (specific activity, 33 Ci/mmol) were obtained from Amer sham Iberica (Madrid, Spain). Sepharose 6B was from Pharmacia Iberica (Madrid, Spain), and the treatment with acid was performed at 50 °C for 3 h with 0.1 N HCl. Ricin and the pokeweed antiviral protein from roots (PAP-R) were generous gifts from Prof. F. Stipe from the Dipartimento di Patologia Sperimentale, Università di Bologna, Bologna, Italy. RPMI 1640 medium was obtained from Life.
Technologies, Inc. NHC epithelial cells were obtained from normal subjects and maintained in our culture collection (19).

Isolation of Ebulin 1—Ebulin 1 was isolated by affinity chromatography on acid-treated Sepharose 6B (AT-Sepharose 6B) at 2-4 °C as follows: 100 g of leaves were cut into small pieces and then ground in a blender and extracted with 800 ml of 280 mM NaCl containing 5 mM sodium phosphate (pH 7.5). The extract was filtered through a cheesecloth, and the fluid was then centrifuged at 25,900 × g for 45 min at 0 °C. The clear supernatant was filtered through a cheesecloth and applied to an AT-Sepharose 6B column (9.5 × 5 cm) preequilibrated with extraction buffer. The column was then washed with the same buffer until absorbance reached the base line. The bound protein was desorbed with elution with 0.2 M D-galactose in the extraction buffer. The peak containing the protein was concentrated with an Amicon YM10 membrane and applied to a Superdex 75 HiLoad column fitted with a FPLC apparatus and equilibrated and eluted with 5 mM sodium phosphate (pH 7.5) containing 0.4 M NaCl. This chromatographic step resolved two main protein peaks, the farthest to the right being ebulin 1 and the largest one being a new D-galactose-binding lectin. The fractions containing the protein peaks were pooled, dialyzed against Milli Q-purified water, and finally freeze-dried.

Analysis of Proteins by SDS-Polyacrylamide Gel Electrophoresis—Analyses of proteins by SDS-polyacrylamide gel electrophoresis were carried out as described by Laemmli (20) using 15% acrylamide gels and the Mighty- Small II system from Hoefer (San Francisco, CA; technical bulletin no. 134). The standards were trypsin inhibitor (M, 20,100), carbonic anhydrase (M, 29,000), alcohol dehydrogenase (M, 37,000), glutamate dehydrogenase (M, 54,000), and bovine serum albumin (M, 68,000).

Polypeptide Synthesis by Eukaryotic and Prokaryotic Translation Systems—In eukaryotic systems translation was coded by endogenous messengers. Preparation of the mammalian cell-free systems and the assays of polypeptide synthesis by rabbit reticulocyte lysates, rat brain, and rat liver, measured as incorporation of ~[35S]methionine into nascent peptides, were performed as described elsewhere (21, 22). In vitro plant protein synthesis was monitored by measuring the incorporation of L-[3H]valine into nascent peptides in wheat germ, Vicia sativa germ, and Cucumis sativus germ translation systems as described previously (23, 24). Preparation of the E. coli ribosomes and high speed supernatant and poly(Phe) poly(U)-directed synthesis was carried out as described elsewhere (25). Incorporation of radioactivity into proteins was assayed by the addition of 0.5 ml of 0.1 N KOH to each reaction mixture. After 10 min, 0.5 ml of 20% (w/v) trichloroacetic acid was added, and the precipitates were collected by filtration on glass fiber filters (Whatman GP/A). Radioactivity was measured by scintillation counting using Ready Safe as scintillation cocktail.

NH2-terminal Sequencing Analysis—Ebulin 1 was subjected to SDS-PAGE in the presence of 2-mercaptoethanol to dissociate A and B chains. Protein electrophoretically purified from poly(vinylidene fluoride) membranes were detected by staining with 0.2% Coomassie Blue R-250 (w/v) in methanol:water:acetic acid (50:40:10) for 0.5–2 min. The membranes were washed in methanol:water:acetic acid (48:47:5), and the protein bands were cut out of the poly(vinylidene fluoride) and applied to a Knauer sequenator with on-line phenylhydantoin-derivatization analyzer according to manufacturer's instructions.

Assay of the 28 S RNA N-Glycosidase Activity—70 µl of rabbit reticulocyte lysate were incubated with 6.8 µg of ebulin 1 or other RIPs for 15 min at 37 °C in a reaction mixture of 80 µl of buffer that contained 30 mM Tris-HCl (pH 7.8), 50 mM KCl, 10 mM dithiothreitol, and 2 mM MgCl2. The reaction was stopped by the addition of 2 µl of 0.5 M EDTA (pH 8.0) and 500 µl of 0.5% SDS containing 50 mM Tris-HCl (pH 7.6). The RNA was extracted by phenolization and ethanol precipitation as described elsewhere (26). The aniline treatment was carried out as follows: 3 µg of RNA were dissolved in 10 µl of water and incubated in the darkness for 10 min with 1 volume of 2 M aniline (pH 4.5). The reaction was stopped by dilution with 200 µl of water and the aniline was removed by two extractions with

![Fig. 2. Analysis of ebulin 1 from S. elbus L. leaves by SDS-polyacrylamide gel electrophoresis.](image-url)

**Fig. 2.** Analysis of ebulin 1 from *S. elbus* L. leaves by SDS-polyacrylamide gel electrophoresis. Ebulin 1 (7.7 µg/well) and the lectin (14 µg/well) were electrophoresed in the presence or the absence of 2-mercaptoethanol as described previously (20). The numbers to the left indicate the corresponding molecular masses of the standards in kilodaltons. The numbers inside the boxes indicate the molecular masses of the corresponding bands in kilodaltons.

![Fig. 3. Effects of ebulin 1 on protein synthesis carried out by mammalian, plant and bacterial cell-free translation systems.](image-url)

**Fig. 3.** Effects of ebulin 1 on protein synthesis carried out by mammalian, plant and bacterial cell-free translation systems. Polypeptide syntheses were performed as is indicated under "Materials and Methods." Controls were run in the absence of inhibitor. Symbols: ○, rabbit reticulocyte lysate; ◊, rat liver; □, rat brain; ▲, *E. coli*; ■, *V. sativa*. The amounts of amino acid incorporated into polypeptides that represent 100% were: rat liver, 0.44 pmol·mg⁻¹ extract protein; rabbit reticulocytes lysate, 0.27 pmol·mg⁻¹ extract protein; *E. coli*, 271 pmol·mg⁻¹ purified ribosomes. In all cases the amount of extract (and ribosomes in the case of *E. coli*) was limiting.

![Fig. 1. Isolation of ebulin 1 from leaves of *Sambucus elbus* L.](image-url)

**Fig. 1.** Isolation of ebulin 1 from leaves of *Sambucus elbus* L. Ebulin 1 was prepared as described under "Materials and Methods." A, affinity chromatography of crude protein extract. Inset, the SDS-polyacrylamide gel electrophoretic analysis (15 µg/well) was carried out as described under "Materials and Methods"; the numbers indicate the molecular masses of the standards in kilodaltons. B, Superdex 75 HiLoad-FPLC of protein from the affinity chromatography step.
**EBULIN**

1, a New Type 2 RIP

**Type 1 RIPS and type 2 RIP- A chains**

Ebulin L A-chain

| IDYPSVSFNLAGAKSTYTIEDFPLKJNL |

Briodin-S

| DVSFRLSATTTSYGVFIKNL |

Trichosanthin R

| DVSFRSLGATTSSYGFPISNL |

TAP 29

| DVSFRSLGATSKKVYPFIISNL |

Luffin a

| DVSFRSLGATSSYGVFPISNL |

MAP 30

| DVSFRSLGATSSYGVFPISNL |

Gelonin

| GDLTVDVSTKAGTYITYVNPFLNL |

Ricin A-chain

| IFKQYPFINFTTAGATVQSYTNFIRA |

Abrin A-chain

| EDRPFKSTECAGTSSQYSKQFPEAL |

Modeccin A-chain

| EDRPFKSTECAGTSSQYSKQFPEAL |

**Type 2 RIP-B chains and lectins**

SNA III

| DGETXAIPEPPIVRIPEGNCLCYDV |

SNA II

| DGETXAIPEPPIVRIPEGNCLCYDV |

Ebulin 1 B chain

| DGETXAIPEPPIVRIPEGNCLCYDV |

Ricin D B chain

| DGETXAIPEPPIVRIPEGNCLCYDV |

**FIG. 4. Amino-terminal amino acid sequences of ebulin 1 and other RIPS.** Amino-terminal amino acid sequence analysis was carried out as described under "Materials and Methods." The box encloses identical amino acids. The sequences of the RIPs were taken from the following references: briodin, Ref. 29; trichosanthin, Ref. 31; TAP 29, Ref. 16; gelonin, Ref. 29; luffin a, Ref. 30; MAP 30, Ref. 15; ricin A chain, Ref. 32; abrin A chain, Ref. 33; modeccin A chain, Ref. 29; SNA 11, Ref. 36; SNA 111, Ref. 35; ricin D-B chain, Ref. 34.

**FIG. 5. rRNA N-glycosidase activity of ebulin 1.** The rRNA N-glycosidase activity was assayed as indicated under "Materials and Methods." Each lane contained 3 μg of RNA. Without treatment with acid aniline: lane 1, control; lane 2, plus ebulin 1; lane 4, plus PAP-R; lane 6, plus ricin. With treatment with acid aniline: lane 3, plus ebulin 1; lane 5, plus PAP-R; lane 7, plus ricin. The arrow indicates the RNA fragment released as a consequence of RIP action upon acid aniline treatment.

**FIG. 6. Preparative SDS-PAGE of total S. ebulus L. crude protein extract.** The RNA was recovered by ethanol precipitation. Electrophoresis of rRNA was carried out in 5% acrylamide gels at 15 mA for 100 min as described elsewhere (27).

**Assays of Toxicity**—The toxicity of ebulin 1 and ricin was evaluated in Swiss mice (average body weight of 30 g) by intraperitoneal injection of variable amounts of protein.

**Assays of Cytotoxicity**—The cytotoxicity of ebulin 1 and ricin was measured by their effects on cellular synthesis of proteins. NHC epithelial cells were allowed to grow in 12-well plates containing RPMI 1640 medium supplemented with 10% calf serum. Variable amounts of inhibitory protein were added and after 18 h at 37 °C the medium was removed and the wells were washed twice with Hanks' balanced salt solution. Then methionine and serum-free medium were added, and the cells were pulse-labeled with 1 μCi/ml L-[35S]methio-

**RESULTS**

During preliminary screening studies on protein synthesis inhibition by plant extracts in our search for RIPS we found that S. ebuelus L. crude protein extracts strongly inhibited protein synthesis and that it agglutinated red blood cells (data not shown). We first attempted to separate both the agglutinin and the translational inhibitory activities by D-galactose affinity chromatography on AT-Sepharose 6B (Fig. 1A). The protein eluting from the column at 0.2 M D-galactose exhibited both red blood cell agglutinin activity and a powerful translational inhibitory activity (data not shown). SDS-PAGE of this protein preparation revealed two protein bands of 68 and 56 kDa that dissociated into three protein bands with apparent Mr, of 34,000, 30,000, and 26,000 in the presence of 2-mercaptoethanol (Fig. 1A, inset). We further attempted to isolate the translational inhibitor. To do so, this protein preparation was chromatographed on Superdex 75 HiLoad FPLC (Fig. 1B). The protein eluted from this column in two major and clearly defined peaks. SDS-PAGE electrophoretic analysis of the two major protein peaks in the absence of 2-mercaptoethanol revealed that peak 1 contained a homogeneous protein with an apparent Mr, of 68,000, while peak 2 gave a homogeneous protein with an apparent Mr, of 56,000 (Fig. 2). In the presence of 2-mercaptoethanol, peak 1 contained only a homogeneous protein band of Mr, 68,000, while peak 2 gave two homogeneous protein bands of Mr, 26,000, A chain, and 30,000, B chain (Fig. 2). The yields of protein of peaks 1 (new lectin) and 2 (ebulin 1) were of 9.2 and 3.2 mg per 100 g of leaves, respectively. Both novel proteins, lectin and ebulin 1, promote full agglutination red blood cells at 182 and 51 μg/ml, respectively.

Ebulin 1 exhibited a strong inhibitory action on mammalian protein synthesis (Fig. 3). The apparent IC50 (protein concen-
tration causing 50% translation inhibition) was found to be 8.5, 15, and 5 ng/ml for rabbit reticulocyte lysates, rat liver, and rat brain, respectively. These values were considerably lower than those reported for type 2 RIPs (5). By contrast, ebulin 1 was inactive against plant protein synthesis in the three systems assayed here, namely, V. sativa (Fig. 3), and C. sativus and wheat germ (data not shown), and also against bacterial protein synthesis with the E. coli cell-free system.

As shown in Fig. 4, amino-terminal sequence analysis of the ebulin 1 A chain revealed a strong sequence homology with type 1 RIPs from Cucurbitaceae in particular with brinodin S (29), luffin a (30), and the anti-HIV 1 proteins trichosanthin R (31) and TAP 29 (16) with 57, 43, 41, and 48% of sequence homology, respectively. Moreover, the ebulin 1 A chain also shares good sequence homology with the A chains from the type 2 RIPs ricin (32) and abrin (33) but less so than with type 1 RIPs from Cucurbitaceae. Sequence homology with type 1 RIPs from other plant families was lower than with RIPs from Cucurbitaceae with the notable exception of gelonin from the Euphorbiaceae Gelonium multiflorum (29). Considering equivalent amino acids, the sequence homology increased slightly. With respect to the B chain, ebulin 1 has a good sequence homology with ricin D and ricin E B chains (34). Surprisingly, the ebulin 1 B chain shared 66 and 70% of sequence homology with SNA III and SNA II respectively, two D-galactose-binding lectins recently isolated from S. nigra L. (35, 36).

The molecular mechanism through which RIPs inhibit protein synthesis is the release of an adenine from a highly conserved rRNA loop of the largest rRNA that is responsible for the interaction of both eukaryotic and prokaryotic elongation factors with the ribosome (1, 37). Treatment of depurinated rRNA with acid aniline releases an RNA fragment which is diagnostic for RIP action (2–4). Accordingly, we studied whether ebulin 1 also exhibits such RNA N-glycosidase activity. As illustrated in Fig. 5, ebulin 1 depurinated the rRNA from rabbit reticulocyte ribosomes which, upon acid aniline treatment of the isolated rRNA, released the diagnostic RNA fragment. As controls, we performed the same experiment with two characteristic RIPs: a type 1 RIP such as the pokeweed antiviral protein from roots (38), and a type 2 RIP such as ricin (2, 6). As expected, all RIPs yielded the diagnostic fragment.

The injection of up to 2 mg of ebulin 1/kg of body weight to mice (average body weight of 30 g), did not undergo lethal toxic effects (Fig. 6). As a control of toxicity we studied the effects of ricin, the classical and highly toxic type 2 RIP (1). In contrast to ebulin 1, ricin injected to mice at 30 μg per kg of body weight was so strongly toxic that 85% of mice died before 48 h. The injection of 0.3 μg of ricin per kg killed mice in less than 12 h. These results suggest that ebulin 1 those not interact in vivo with ribosomes. To gain insights on this hypothesis we studied the cytotoxic effects of ebulin 1 on NHC epithelial cultured cells and compared them with dose displayed by ricin. As Fig. 6 illustrates, ebulin 1 was without effect on cell protein synthesis measured as incorporation of L-[35S]methionine into proteins even at 6 μg/ml. In contrast, ricin inhibited cell protein synthesis with an IC50 of 1.9 ng/ml which is in close agreement with previous reports that used different cells (1, 5).

**DISCUSSION**

We have found ebulin 1, a new type 2 RIP, in a preparation of protein from *S. ebulus* leaves obtained using affinity chromatography on AT-Sepharose 6B, indicating that ebulin 1 binds to D-galactose. Our data suggest that ebulin 1 belongs to the type 2 RIP category since: (a) the inhibitory effects on protein synthesis are exerted at extremely low concentrations (subnanomolar range); (b) the size of both the A and B chains of ebulin 1 is roughly equivalent to the size of the A and B chains of all the type 2 RIPs hitherto known, namely ricin, abrin, modeccin, viscumid, and volkensin (reviewed in Ref. 39); (c) ebulin 1 shows N-glycosidase activity against mammalian ribosomes which, after treatment of the isolated rRNA with acid aniline, release the Endo’s diagnostic RNA fragment; (d) the amino-terminal amino acid sequences of both the A and B chains display good sequence homology with type 1 RIPs and with the A and B chains from type 2 RIPs. Ebulin 1 also fulfills other minor RIP properties (data not shown). It is noteworthy that the disulfide bridge(s) that maintain(s) both chains linked is(are) highly sensitive to 2-mercaptoethanol. This was so much so that the electrophoresis minus and plus reductant must be conducted in distant wells on the same plate or on separate plates for good results. Taking into account that some type-2 RIPs also display lower IC50 values when dissociated in the A plus B chains (1) and that the translation assays are carried out in the presence of dithiorhethitol, it can be assumed that the extremely low IC50 of ebulin 1 would be due to cleavage of the disulfide bond(s).
Ebulin I, a New Type 2 RIP

Thus, the apparent IC₅₀ values of ebulin I fit in well with those reported for the other type 2 RIPS in dissociation conditions, especially ricin, abrin, and volkensin (39). In agreement with what has been observed for other type 2 RIPS (4, 40), ebulin I did not inhibit in vitro plant or bacterial protein synthesis. This is of special importance for the molecular cloning and expression of the ebulin I gene. Recent studies have shown that some type 1 RIPS inhibit protein synthesis in E. coli, thus hindering the expression of their genes in recombinant bacteria (34, 41).

A very important feature concerning ebulin I is the lack of toxicity both on cells and intact animals. Since the dimer did not dissociate in the culture media under our experimental conditions (data not shown), a phenomenon that would hinder the A chain translocation through plasma membrane (6), our data suggest that ebulin I does not reach the ribosomes in intact cells perhaps due to defects in the intracellular routing of this kind of RIPS (6, 42, 43). Among them, a premature degradation, an inability to translocate through the endosomal membrane or a difficulty to reduce the disulfide bridge(s) that links the A and B chains, could be considered. Alternatively, the lack of toxicity could be a consequence of the association of the dimer yielding inactive oligomers unable to display the powerful activity of the isolated A chains. The lack of toxicity of intact ebulin I even at high concentrations makes it highly interesting for immunotoxin construction since it will not show harmful residual toxicity as ricin does, and that greatly restricts the use of ricin A chain purified from castor beans (5).

In a preliminary study we also found rRNA N-glycosidase and translational inhibitory activities (most probably type 2 RIPs, since they are bound to AT-Sepharose 6B) in several crude preparations of lectins from different plant families (Caprifoliaceae) are opened to research on immunodeficiency virus type I for the potential treatment of AIDS. Recent studies have shown that some type 1 RIPs lack of toxicity of intact ebulin I even at high concentrations makes it highly interesting for immunotoxin construction since it will not show harmful residual toxicity as ricin does, and that greatly restricts the use of ricin A chain purified from castor beans (5).

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REFERENCES

Technology 10, 405-412
8. Lambert, J. M., Blattner, W. A., McIntyre, G. D., Goldschmied, V. S., and 
12. Ebulin 1 shares all the basic functional and structural properties of all type 1 RIPs hitherto known (ricin, abrin, and volkensin; Refs. 1 and 5). With all these findings, a very interesting plant genus Ebulin 1 is the lack of toxicity, its usefulness in the construction of immunotoxins for therapy (7- 
9), 1 particularly the lack of toxicity, its usefulness in the construction of immunotoxins for therapy (7- 
9), especially when the anti-A chain antibodies appear in long 