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A ribosomal protein is specifically recognized by saporin, a plant toxin which inhibits protein synthesis

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Many plants express enzymes which specifically remove an adenine residue from the skeleton of the 28 S RNA in the major subunit of the eukaryotic ribosome (ribosome inactivating proteins, RIPs). The site of action of RIPs (A4324 in the rRNA from rat liver) is in a loop structure whose nucleotide sequence all around the target adenine is also conserved in those species which are completely or partially insensitive to RIPs. In this paper we identify a covalent complex between saporin (the RIP extracted from *Saponaria officinalis*) and ribosomal proteins from yeast (*Saccharomyces cerevisiae*), by means of chemical crosslinking and immunological or avidin–biotin detection. The main complex (mol. wt. ≃ 60 kDa) is formed only with a protein from the 60 S subunit of yeast ribosomes, and is not detected with ribosomes from *E. coli*, a resistant species. This observation supports the hypotesis for a molecular recognition mechanism involving one or more ribosomal proteins, which could provide a 'receptor' site for the toxin and favour optimal binding of the target adenine A4324 to the active site of the RIP.

Ribosome inactivating protein; Molecular recognition; Crosslinking

1. INTRODUCTION

Protein synthesis requires the interaction of many soluble proteins and cofactors with the ribosome, which is also the target of many natural toxins and synthetic drugs. Ribosome inactivating proteins (RIPs) constitute a biologically and pharmacologically interesting class of proteins which are expressed by several plants and irreversibly damage eukaryotic ribosomes at the EF2 binding site [1–5]. A typical RIP is a specific *n*-glycosidase, which removes the adenine residue at position 4324 (for rat rRNA) from the skeleton of the 28 S rRNA; this mechanism is responsible for the toxic activity of the RIPs tested so far (ricin A chain [6]; the RIP from barley seeds [7]; gelonin, momordin, saporin and PAP [8]). The enzymatic efficiency of these toxins is very high, as demonstrated by K_m values of 0.1 μM and a turnover number of 1,500/min calculated for ricin A chain [9]; thus they have been considered ideal for the preparation of 'immunotoxins', carrying the active (A) chain of a RIP covalently bound to a monoclonal antibody [10–12]. Such immunotoxins have been used to selectively kill neoplastic cells [13].

The efficiency of RIPs is 10,000-fold higher with intact ribosomes than with isolated rRNA [14]. Moreover, selective removal of proteins from the 60 S ribosomal subunit can reduce the efficiency of RIPs [15]. Finally, ribosomes from different species have different sensitivities to RIPs, given that those of prokaryotes [16] and some from eukaryotes [15] are completely or partially unaffected, even though the rRNA sequence around A4324 (attacked by the toxin) is conserved. These findings would be explained if one or more specific contacts between RIPs and ribosomal proteins were necessary for the formation of a productive Michaelis complex. Indeed, the sequence and organization of ribosomal proteins is more variable than the sequence of nucleotides at the target site of RIPs.

In this paper we show, by crosslinking experiments, the formation of a covalent complex between saporin (the RIP extracted from the seeds of *Saponaria officinalis*, mol. wt. 30 kDa) and (at least) one ribosomal protein from the 60 S subunit of yeast ribosomes. We believe that this complex (mol. wt. ≃ 60 kDa) proves the presence of protein–protein interactions in the formation of a productive Michaelis complex between the toxin and the ribosome. The specific nature of the complex is supported by the observations that (i) it is not detected with *E. coli* ribosomes and (ii) it is not affected by the crosslinker used and the detection method employed.

2. MATERIALS AND METHODS

Saporin was prepared and purified as described by Stirpe et al. [17] and stored at −80°C in 10 mM phosphate buffer, pH 7.0. The reaction with bromoacetic acid *N*-hydroxysuccinimide ester, NSBr [18], was carried out by incubation with a 40-fold excess of the reagent in 10 mM

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phosphate buffer, pH 7.0, for 24 h at 4°C. The reaction was stopped by the addition of glycine and excess reagent was removed by dialysis against 10 mM HEPES, pH 7.4, containing 50 mM KCl and 5 mM magnesium acetate (HKM buffer), or by gel filtration with Sephadex G25 (Pharmacia) equilibrated with the same buffer. The inhibitory activity of NSBr-saporin, tested on a rabbit reticulocite lysate (Promega), was the same as that of native saporin. Saporin was also modified with a different crosslinker, Dithiobis[succinimidyl]propionate, following the method described by Lomant [19].

Ribosomes and their heavy and light subunits from yeast (*Saccharomyces cerevisiae*) were prepared following standard procedures [20] and stored in 50% glycerol at −80°C in HKM buffer. Rabbit anti-saporin antiserum was prepared following a standard protocol [21].

The crosslinking reaction was initiated by mixing ribosomes at a final concentration of 5–10 mg/ml with a stoichiometric amount of NSBr-modified saporin in HKM buffer. After incubation at room temperature for 2 h, the reaction mixture was loaded on a centrifuge tube containing a 40% sucrose solution in HKM buffer and centrifuged for 16 h at 40,000 rpm and at 4°C. The ribosome-containing pellet was resuspended and analyzed by SDS electrophoresis following Laemmli [22]. Ribosomal proteins were transferred on a PVDF membrane (Immobilon P, Millipore) and a goat anti-rabbit antibody conjugated with peroxidase was used to reveal the presence of saporin. In order to use a second method for detection, saporin was modified with biotin–hydrazide at the C-terminal residue [23], before reaction with NSBr, and revealed by avidin (Sigma) conjugated with peroxidase.

3. RESULTS AND DISCUSSION

Saporin has a high ribosome-inactivating activity in vitro but low toxicity in vivo because, contrary to ricin, it lacks subunit B which is responsible for the binding and internalization of the toxin [24]; therefore it was possible to obtain a high titre rabbit antiserum for immunodetection. Moreover, the absence of cysteines in its sequence [25] is another useful feature which prevents the formation of saporin dimers after modification with the crosslinker NSBr (which reacts with primary amines and sulphydril groups).

Addition of NSBr-modified saporin to yeast ribosomes generates at least one new band which we believe to be a covalent complex with one ribosomal protein. A typical experiment is reported in Fig. 1A, which shows a Western blot of the SDS electrophoretic pattern of ribosomal proteins from ribosomes treated with NSBr-modified saporin, and for comparison from controls. The new sharp band which is more clearly detected in reacted samples has a mol. wt. of ≈ 60 kDa, higher than that of other bands (35–60 kDa), some of which are barely visible and are probably due to the

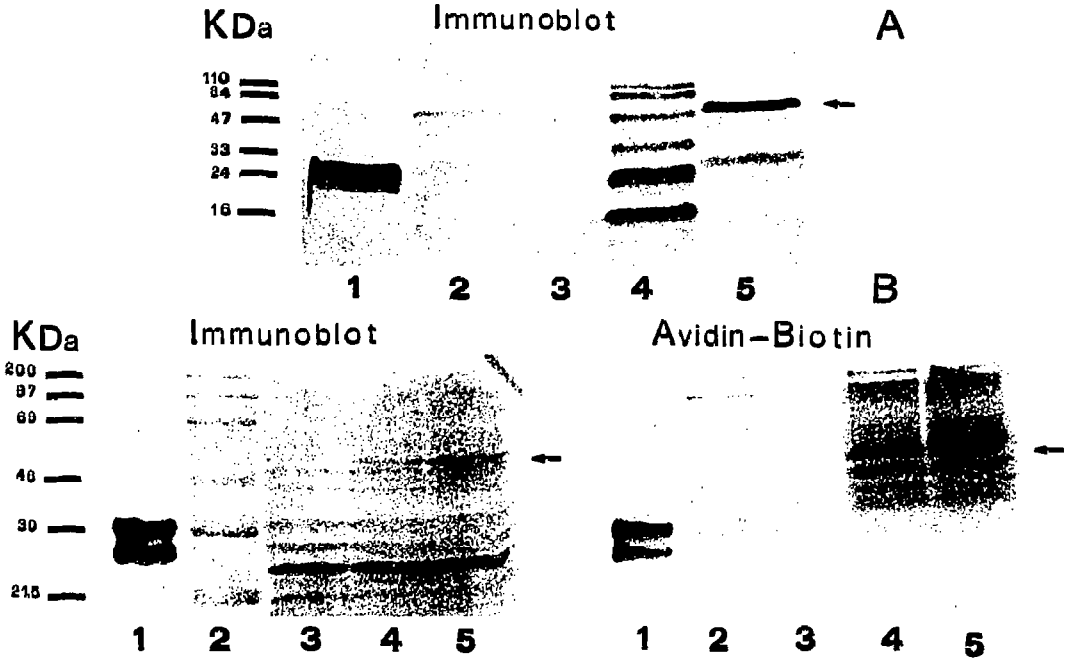

Fig. 1. Western blot analysis of ribosomal proteins after SDS-PAGE. (A) (lane 1) NSBr-saporin; (lane 2) yeast ribosomes treated with NSBr-saporin; (lane 3) yeast ribosomes treated with native saporin, (lane 4) mol. wt. standards (pre-stained, Biorad) (lane 5) sample as in lane 2 (higher concentration). Saporin activated with NSBr was allowed to react with ribosomes and the membrane of this experiment was incubated with a rabbit anti-saporin antiserum (1/1,000 dilution) and developed with goat anti-rabbit IgG (1/3,000 dilution) conjugated with peroxidase. (B) (lane 1) NSBr–saporin modified with biotin–hydrazide; (lane 2) mol. wt. standards (pre-stained, Amity); (lane 3) yeast ribosomes treated with native saporin; (lanes 4 and 5) yeast ribosomes treated with NSBr–saporin modified with biotin–hydrazide. NSBr-saporin was modified at the C-terminus with biotin–hydrazide before the reaction with ribosomes; this modification may be responsible for the heterogeneous SDS pattern (lane 1, left and right panels) possibly because of the multiple binding of biotin. The membrane of the experiment in the left panel was treated as in A, while that of the experiment in the right panel was incubated and developed with avidin (Bio-Rad) conjugated with peroxidase (1/1,000 dilution). The band corresponding to the covalent complex between saporin and a ribosomal protein (mol. wt. 60 kDa) is indicated by an arrow in all three experiments of this figure. Some other bands, sometimes clearly evident in the gel, are visible when detection is carried out with rabbit and goat antisera; most of these bands correspond to a mol. wt. < 30 kDa and are attributed to cross-reactivity of the IgG, as demonstrated by the fact that they are not visible at all when avidin is used as the revealing agent (B, right panel). Finally it should be noted that bands with mol. wt. 35–60 kDa are detected only in ribosomes treated with NSBr–saporin, while they are not present in control ribosomes.

cross-reactivity of the goat anti-rabbit antiserum. Since the activity of NSBr–saporin is virtually unchanged with respect to native saporin, we believe that its interaction with the ribosome is directed towards the natural target (adenine residue in 28 S rRNA). The same band, though with a lower yield, has been detected when saporin, modified with a different cross-linker (dithiobis[succinimidyl]propionate) [19], was added to yeast ribosomes (data not shown).

As a test of specificity, the same experiment has been carried out using a different saporin derivative, modified at the C-terminal residue with biotin–hydrazide before conjugation with the crosslinker NSBr; in the latter case, avidin conjugated with peroxidase has been used to stain the SDS gel. In Fig. 1B the pattern observed with the biotin–avidin technique (right panel) is compared with that revealed by the immunological technique (left panel); again the more intense of the new bands is 60 kDa, indicating a 1:1 covalent complex with a 30 kDa ribosomal protein.

Fig. 2 shows a Western blot of ribosomal proteins obtained from separated heavy and light subunits of yeast ribosomes after reaction of NSBr-modified saporin. The experiment indicates that the 60 kDa covalent complex is formed only with proteins from the 60 S subunit, as expected, because RIPs are active on the 28 S rRNA.

It has been reported [26] that three different classes of proteins can be 'stripped' by washing ribosomes with a buffer at high ionic strength, or at low Mg concentration, or both. This treatment, which removes from the ribosome some 'exchangeable' proteins, may either destroy the site recognized by RIPs or expose new sites. Therefore we carried out the same reaction in 5 mM phosphate buffer, pH 7.0, containing 1 mM magnesium

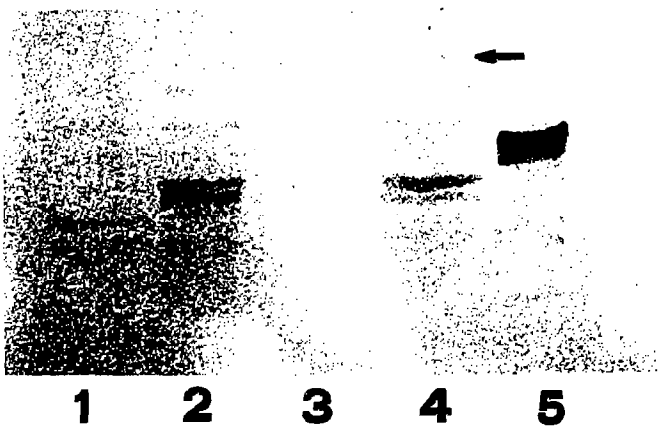

Fig. 2. Western blot analysis of ribosomal proteins after SDS-PAGE. (lane 1) yeast 40 S subunit from native ribosomes; (lane 2) yeast 60 S subunit from native ribosomes; (lane 3) yeast 40 S subunit from ribosomes treated with NSBr–saporin; (lane 4) yeast 60 S subunit from ribosomes treated with NSBr–saporin; (lane 5) NSBr–saporin. Immunodetection was carried out using of a rabbit anti-saporin antiserum (1/1,000 dilution) and goat anti-rabbit IgG (1/3,000 dilution) conjugated with peroxidase.

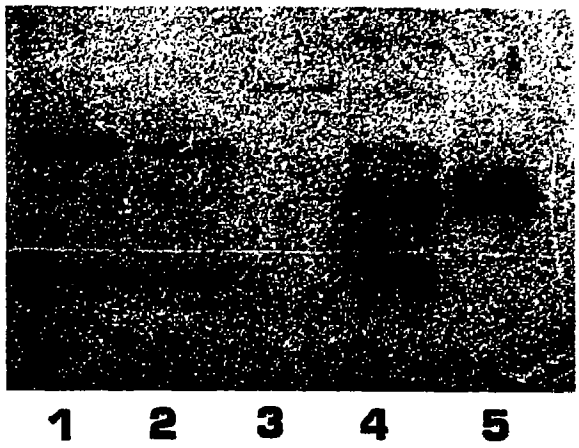

Fig. 3. Western blot analysis of ribosomal proteins after SDS-PAGE. (lane 1) *E. coli* ribosomes treated with native saporin; (lane 2) *E. coli* ribosomes treated with NSBr-saporin; (lane 3) complex between saporin and yeast ribosomal proteins eluted from a previous gel and re-analyzed by SDS-PAGE; (lane 4) mol. wt. standards (pre-stained, Bio Rad) (lane 5) NSBr-saporin. Immunodetection was as in Fig. 2.

acetate; thereafter the ribosomes were pelletted in the same buffer containing 0.15 M KCl. The corresponding electrophoretic pattern, revealed using either the immunological or the avidin–biotin technique, is the same as that in Fig. 2, and shows the presence of a main complex of mol. wt. 60 kDa, and some minor bands at lower molecular weights.

It has been observed that ricin A chain binds Cibachron blue F3GA stoichiometrically [27], possibly at the level of the active site. When the crosslinking reaction with NSBr-modified saporin was carried out in the presence of this dye, the same pattern was observed; this indicates that the stability of the saporin–ribosome Michaelis complex is sufficiently high to displace Cibachron blue from the active site (even when the reaction was carried out at fairly high dye concentrations).

Finally, we carried out an identical crosslinking experiment with NSBr-modified saporin added to ribosomes from *E. coli*, which is known to be resistant to RIPs [16]. The results reported in Fig. 3 show that, in this case, no covalent complex was detected.

4. CONCLUSIONS

Chemical crosslinking of saporin to yeast ribosomal proteins suggests that the molecular mechanism by which the toxin recognizes its target is mediated by protein–protein as well as protein–RNA interactions, and that the Michaelis complex is probably stabilized by molecular contacts over and above the binding of rRNA/A4324 to the active site of the RIP. The result of the experiment reported in this paper is that at least one ribosomal protein (mol. wt. 30 kDa) from the 60 S subunit comes into contact with saporin and is crosslinked. The hypothesis that at least one ribosomal

protein is involved in the recognition by the toxin is fully consistent with the observation that RIPs are more active on the whole ribosome than on isolated rRNA [14]. The absence of a covalent complex when *E. coli* ribosomes were challenged with NSBr-modified saporin, suggests that ribosomes from some organisms are resistant because of a lower stability of the Michaelis complex and the lack of specific protein–protein interactions in the recognition site. Since alternative interpretations of our results are possible (e.g. the absence of a sulphydryl at suitable distance from the crosslinker), this should be taken as a working hypothesis, but we believe it is a reasonable and challenging interpretation of the specificity of toxin action. Finally it should be noted that more than one ribosomal protein may be involved in the surface of contact with saporin, since minor bands were often detected in the Western blot (Fig. 1). However the 60 kDa complex was observed with whole ribosomes and 60 S subunits, and it was detected using two crosslinkers and two different staining techniques. Further work is in progress to extend the same type of experiment to other RIPs (notably the A chain of ricin), and particularly to isolate and sequence the ribosomal protein(s) involved in the interaction with saporin.

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