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The effects of *N*-glycosylation on the lectin activity of recombinant ricin B chain*,†

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ABSTRACT

Soluble, biologically-active recombinant ricin B chain has been produced by expressing B chain-encoding DNA in heterologous eukaryotic or prokaryotic hosts. *N*-Glycosylated recombinant ricin B chain expressed in *Xenopus* oocytes bound to both immobilized asialofetuin and immobilized lactose. Non-glycosylated ricin B chain expressed in either *E. coli* or in tunicamycin-treated oocytes did not bind to immobilized lactose. However, it did bind to asialofetuin, and increasing concentrations of free lactose did not reduce this asialofetuin binding dramatically, in contrast to the effect of free lactose on the binding of either glycosylated recombinant B chain or native ricin B chain.

INTRODUCTION

The B chain of the heterodimeric plant cytotoxin ricin, which occurs in castor oil (*Ricinus communis*) seeds, is a D-galactose-specific lectin[1]. Amino acid sequence analysis has shown that ricin B chain is a product of gene duplication[2], and the X-ray structure has confirmed that the polypeptide folds into two separate globular domains, each of which binds to D-galactopyranoside[3]. The protein is also glycosylated, having two *N*-linked oligosaccharide side-chains[1]. The gene encoding the ricin precursor has been cloned[4,5], and the individual ricin A and B chains have been expressed in a variety of heterologous systems[6–12].

Recombinant ricin B chain (rRTB) may be assayed for biological activity by its ability to bind D-galactose or lactose[9–12]. We have taken advantage of this property to purify rRTB by affinity chromatography on immobilized lactose (Selectin-2) columns. We report herein that whereas rRTB produced in both *Xenopus* oocytes (which is *N*-glycosylated) and in *E. coli* (which is not) did bind to the immobilized glycoprotein asialofetuin (which contains D-galactosyl terminal groups), only the former was able to bind to immobilized lactose. To examine whether the absence of glycosylation in the context of *E. coli* production of rRTB contributes to its failure to bind lactose, we have used tunicamycin-treated oocytes to produce nonglycosylated rRTB in the eukaryotic

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host. The results we obtained indicated that the absence of *N*-glycosylation accounts for the failure of rRTB to bind lactose and, further, it reduces the ease with which rRTB bound to asialofetuin can be displaced by free lactose.

EXPERIMENTAL

Production of recombinant proteins in E. coli. — Nonglycosylated rRTB was produced in *E. coli* and targeted to the periplasmic space by use of the expression vector pIN-III-Omp A3 exactly as described previously[12]. Nonglycosylated proricin was produced by ligating a proricin-encoding sequence (from base + 8 according to the numbering scheme used by Lamb *et al.*[4]) on a *Hind* III–*Sal* 1 fragment into pIN-III-Omp A2[13], also restricted with *Hind* III and *Sal* 1, to generate pIN 617. The junction across the 3′-end of the Omp A signal sequence (within vector DNA) and the start of the proricin sequence, together with a short stretch of intervening polylinker sequence and the amino acids this sequence encodes, is as shown in Scheme 1.

Signal cleavage site	*Hind* III	(Start of proricin sequence)
↓	↓	↓

5′ … GCT GAA TTC CAA GCT TGG ATC GAG GCC AAA … 3′
Ala Glu Phe Gln Ala Trp Ile Glu Ala Lys

Scheme 1.

Recombinant plasmids were introduced into *E. coli* JA221 (1pp$^-$, hsdM$^+$, trpt5, leu B6, lac Y, rec A1, F′lacI^q, lac$^+$, and pro$^+$) by $CaCl_2$-mediated transformation. Bacteria were grown at 18° in the presence of ampicillin (50 $\mu g \cdot mL^{-1}$) and mM isopropyl β-D-thiogalactoside in defined medium[12]. Cultures were grown to an A_{550} of 0.6 to 0.8 and the cells were harvested by centrifugation. The periplasmic fraction was prepared as described previously[12].

Synthesis of prericin B chain transcripts and microinjection. — Prericin B chain transcripts were synthesized *in vitro* in the presence of the capping dinucleotide 7-Me (5′) G ppp G (5′) OH and T7 RNA polymerase as described earlier[10]. Purified RNA was dissolved in distilled water at a concentration of 1000 $\mu g \cdot mL^{-1}$. Microinjection into batches of 100 oocytes from *Xenopus laevis*, pulse labelling with [^{35}S]-methionine, and oocyte homogenization were performed exactly as described previously[10]. To prevent *N*-glycosylation, oocytes were injected with tunicamycin (Sigma) at 40 $\mu g \cdot mL^{-1}$ and incubated in Barth's medium containing 2 $\mu g \cdot mL^{-1}$ of tunicamycin for 24 h before subsequent RNA injections. This preincubation with tunicamycin has previously been shown to be essential in order to exhaust the endogenous pool of dolichol-linked oligosaccharide side-chain precursors[14].

Asialofetuin binding. — The ability of proteins to bind to immobilized asialofetuin was assayed by use of a modification of a procedure described previously[15]. Briefly, asialofetuin was used to coat the wells of a microtitre plate, samples containing RTB were added, and, after washing, biologically active RTB bound to the asialofetuin was measured by adding rabbit anti-RTB antibodies, followed by ^{125}I-labelled protein A. A calibration curve was prepared with native RTB, and this immunodetection assay was used to quantitate rRTB.

Lactose binding. — The ability of proteins to bind to immobilized lactose was determined by passing cellular homogenates (1 mL) down a small column containing 1 mL of Selectin-2 beads (Pierce). For *E. coli* preparations, the column was pre-equilibrated in 10mM Tris·HCl, pH 7.2, and protein samples were applied in this buffer. For oocyte samples, the columns were equilibrated in oocyte homogenization buffer [100mM NaCl, 20mM Tris·HCl, pH 7.6, and 1% (v/v) Triton X-100]. Each column was washed with appropriate buffer until no further protein emerged. Bound material was eluted by adding a series of 1-mL aliquots of buffer containing increasing concentrations of lactose (0 to 50mM).

Other methods. — Published procedures were followed for immunoprecipitation[16], sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis[16], enzymic deglycosylation using endo-*N*-acetyl-β-D-glucosaminidase H[17], lentil lectin-Sepharose chromatography[16], and Western blotting[18]. Blots were probed with polyclonal antibodies raised in rabbits against RTB purified from *Ricinus communis* seeds[19], and were developed with the biotinylated protein A–streptavidin-peroxidase method or with ^{125}I-labelled protein A, followed by fluorography[11].

RESULTS AND DISCUSSION

We have previously produced rRTB in *E. coli*. The DNA encoding RTB was fused to that encoding the *E. coli* Omp A signal peptide by use of the expression-secretion vector pIN-III-Omp A3[13]. The rRTB accumulated in a soluble form in the periplasmic space, and was biologically active in that it bound to immobilized asialofetuin[12]. In the present study, we passed periplasmically-targeted rRTB produced in *E. coli* down an immobilized lactose (Selectin-2) column. The rRTB passed straight through the Selectin-2 column (Fig. 1, lane 2). rRTB was not eluted from the column by free lactose (Fig. 1, lane 1) and was not detected still bound to the column (data not shown). During ricin biosynthesis in *Ricinus communis* seeds, RTB is initially synthesized together with ricin A chain as part of a single precursor polypeptide[19]. We have previously expressed this precursor, proricin, in *Xenopus* oocytes[10]. The recombinant proricin, which was segregated into the oocyte endoplasmic reticulum, was core-glycosylated, soluble, and able to bind to both lactose and asialofetuin. In the present study we have expressed proricin in *E. coli*, once again utilizing the Omp A signal peptide to direct the recombinant product to the periplasmic space. The recombinant proricin bound to asialofetuin (data not shown), but it did not bind to lactose (Fig. 1, lane 6). One possible explanation for these observations was that the absence of

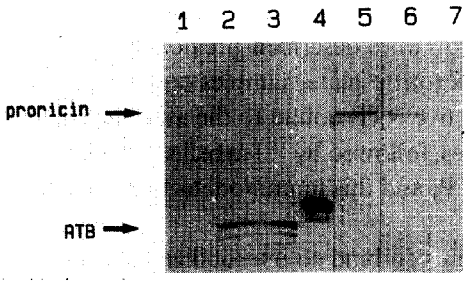

Fig. 1. Nonglycosylated RTB and proricin do not bind to immobilized lactose. The periplasmic fractions from *E. coli* cells expressing either rRTB or proricin were passed down a Selectin-2 column. Various fractions were separated by sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis, blotted onto nitrocellulose, and probed with rabbit anti-RTB antibodies: Lane 1, material eluted with 50 mM lactose from an rRTB-loaded column; lane 2, rRTB which passed straight through the column; lane 3, rRTB in the periplasmic fraction applied to the column; lane 4, purified glycosylated RTB standard; lane 5, proricin in the periplasmic fraction; lane 6, proricin which passed straight through the column; and lane 7, proricin eluted from the column with lactose. The minor band visible in lanes 2 and 3 may have resulted from partial proteolysis of the rRTB or from nonspecific antibody binding.

glycosylation prevented the recombinant products from binding lactose. Thus rRTB or proricin produced in the eukaryotic host were *N*-glycosylated and bound to both lactose and asialofetuin. To further explore this possibility, we produced nonglycosylated rRTB in the eukaryotic host. The 5′-signal sequence and the RTB-coding sequence were excised from preproricin cDNA and fused in frame to generate pre-RTB cDNA. The pre-RTB cDNA was transcribed *in vitro* and the pre-RTB transcripts were microinjected into *Xenopus* oocytes[10]. Where appropriate, the oocytes had been pretreated with tunicamycin to prevent *N*-glycosylation. Recombinant RTB produced in tunicamycin-treated oocytes had an apparent molecular mass of 30 kDa (Fig. 2, lanes 1 and 4), and

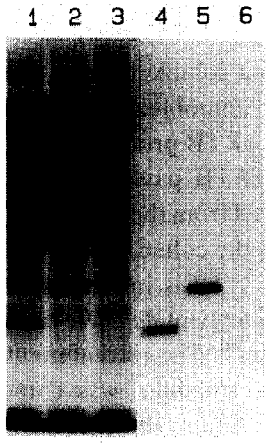

Fig. 2. Expression of RTB in *Xenopus* oocytes. Lanes 1–3 show total [^{35}S]methionine-labelled proteins synthesized by oocytes which had been microinjected with an *in vitro* transcript encoding preRTB (lane 2), tunicamycin-treated oocytes injected with preRTB (lane 1), or oocytes mock injected with water (lane 3). Lanes 4–6 show the products immunoprecipitated from the total products, shown in lanes 1–3, respectively, by use of rabbit anti-RTB antibodies.

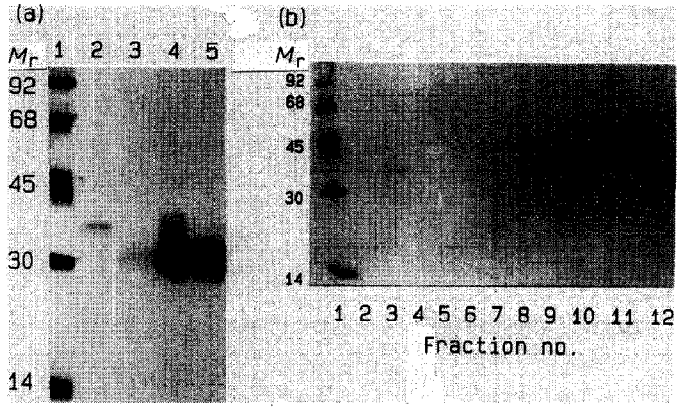

Fig. 3. RTB produced in oocytes is *N*-glycosylated. (a) rRTB produced in control (lanes 2 and 3) or tunicamycin-treated (lanes 4 and 5) oocytes before (lanes 2 and 4) and after (lanes 3 and 5) incubation with endo-*N*-acetyl-*β*-D-glucosaminidase H; lane 1, molecular-mass markers. (b) rRTB produced in oocytes bound to a lentil lectin-Sepharose column and was eluted by adding 100mM D-mannose where indicated by the arrow.

that produced in untreated oocytes had an apparent molecular mass of 34 kDa (Fig. 2, lanes 2 and 5). Both recombinant products were segregated into the oocyte endomembrane system (data not shown). The apparent molecular-weight difference resulted from *N*-glycosylation since (a) treatment of the 34-kDa rRTB with endo-*N*-acetyl-D-glucosaminidase H reduced its apparent molecular mass to 30 kDa (Fig. 3a), and (b) the 34-kDa rRTB, in contrast to the 30-kDa rRTB produced in tunicamycin-treated oocytes, bound to lentil lectin-Sepharose and could be eluted with D-mannose (Fig. 3b).

N-Glycosylated rRTB produced in oocytes bound to a Selectin-2 column and was eluted from the column by free lactose, whereas nonglycosylated rRTB produced in the same host did not (Fig. 4).

Both glycosylated rRTB, produced in oocytes, and nonglycosylated rRTB, produced in either tunicamycin-treated oocytes or in *E. coli*, bound to asialofetuin (Fig. 5). However, whereas glycosylated rRTB (in common with native ricin) showed signif-

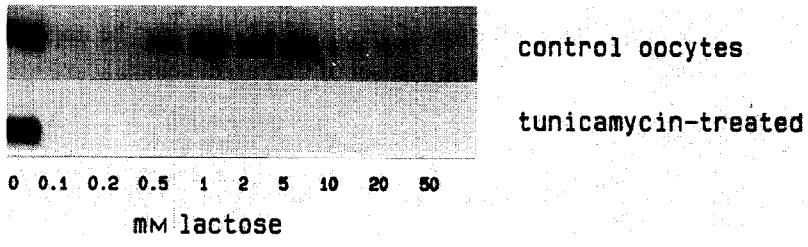

Fig. 4. Binding of rRTB to immobilized lactose. *N*-Glycosylated rRTB produced in control oocytes and nonglycosylated rRTB produced in tunicamycin-treated oocytes were passed down a 1-mL Selectin-2 column. The track label 0 (mM lactose) shows rRTB recovered by immunoprecipitation from the appropriate oocyte homogenate. Other tracks show bound rRTB which was eluted from the column with increasing lactose concentrations and recovered by immunoprecipitation. In the case of the tunicamycin-treated oocytes, all the rRTB passed straight through the column.

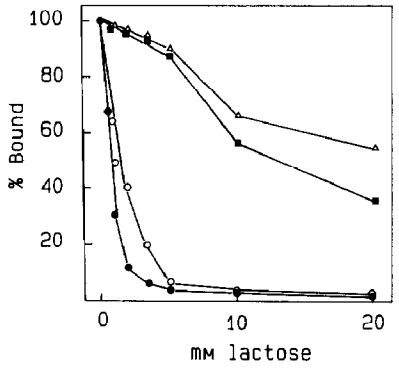

Fig. 5. Binding of rRTB to immobilized asialofetuin. *N*-Glycosylated rRTB produced in oocytes (-○–○-) and nonglycosylated rRTB produced in either tunicamycin-treated oocytes (-■–■-) or *E. coli* (-△–△-) was added to immobilized asialofetuin in the presence of increasing concentrations of free lactose, and the proportion which bound was determined and compared to native ricin (-●–●-).

icantly reduced asialofetuin-binding in the presence of increasing concentrations of free lactose, lactose had much less effect on the binding of nonglycosylated rRTB (Fig. 5).

The failure of nonglycosylated rRTB to bind lactose does not result from the production of grossly misfolded or aggregated recombinant proteins. Nonglycosylated rRTB released from the periplasmic space of *E. coli* or the endomembrane system of *Xenopus* oocytes appears to be soluble and stable. Furthermore, nonglycosylated rRTB was still a biologically active lectin which effectively bound to asialofetuin.

There are several examples known where the extent of glycosylation affects the biological activity of a polypeptide. One such example is the IgE-binding protein which either suppresses or potentiates the IgE response, depending on the extent to which it is glycosylated[20,21].

An earlier study indicated that the oligosaccharide side-chains could have a role in the cytotoxic action of ricin[22]. The oxidation of D-mannose residues by periodate treatment appeared to change the conformation of ricin and to greatly reduce its cytotoxicity. Since the modification did not affect the ability of ricin to bind to the target-cell surface, the ability to inactivate ribosomes, or the toxins resistance to proteolytic degradation, the native conformation of glycosylated ricin may be important for the penetration of ricin A chain into the cytosol of the target cells[22].

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