Restoration of lectin activity to a non-glycosylated ricin B chain mutant by the introduction of a novel N-glycosylation site

Jinbiao Zhan, Michelle de Sousa, John A. Chaddock, Lynne M. Roberts, J. Michael Lord*

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, UK

Received 18 February 1997; revised version received 18 March 1997

Abstract Ricin B chain (RTB) is an N-glycosylated, galactose-specific lectin. Removal of the two native N-glycosylation sites at Asn^{95} and Asn^{135} by site-directed mutagenesis generated a recombinant protein devoid of lectin activity. Two novel N-glycosylation sites were introduced into RTB at Asn^{42} and Asn^{123}, either singly or in combination. Microinjection of pre-RTB transcripts into *Xenopus* oocytes showed that these novel sites became glycosylated in vivo. The single oligosaccharide site chain at Asn^{42} restored lectin activity to RTB, whereas glycosylation at Asn^{123} or simultaneous glycosylation at Asn^{42} and Asn^{123} failed to do so.

Key words: Ricin B chain; Lectin activity; N-glycosylation

1. Introduction

Ricin is a heterodimeric glycoprotein in which a toxic polypeptide (the A chain or RTA) is joined by a disulfide bond to a cell-binding polypeptide (the B chain or RTB) [1]. RTB is a galactose-specific lectin and both RTA and RTB are glycosylated, each containing two N-glycosylation sites [2]. The X-ray crystallographic structure of ricin has shown RTB to be a bilobal molecule consisting of two distinct globular domains (1 and 2) with identical folding topologies [3], with each domain in turn containing three subdomains (α, β and γ, respectively). Each subdomain appears to have been derived from the same ancestral galactose-binding peptide [4]. Co-crystallisation of ricin in the presence of lactose demonstrated that only subdomains 1α and 2γ retain significant sugar binding activity [5], although a more recent biochemical and mutational analysis of RTB indicates that subdomain 1β may also have lectin activity [6].

We have produced correctly glycosylated, biologically active RTB by microinjecting pre-RTB transcripts into *Xenopus* oocytes [7]. In a mutational analysis of recombinant RTB, we found that a non-glycosylated mutant in which both of the Asn residues that normally carry N-linked oligosaccharides had been changed to Gln was devoid of lectin activity [8]. To further establish the importance of strategically positioned N-linked oligosaccharide side chains for lectin activity, we have introduced two novel N-glycosylation sites into RTB close to the abrogated native sites. Here we report that one of these novel introduced glycosylation sites restores lectin activity to RTB.

*Corresponding author. Fax: (44) 1203-523-701.
E-mail: ml@dna.bio.warwick.ac.uk

2. Materials and methods

2.1. Mutagenesis

Mutagenesis of RTB cDNA was performed by standard methods using an oligonucleotide-directed in vitro mutagenesis kit (Amersham) as instructed by the manufacturer. Mutations were verified by dideoxy sequencing. Mutagenic oligonucleotides were synthesised on an Applied Biosystems Model 380B DNA synthesizer.

2.2. Synthesis and expression of pre-RTB transcripts

Pre-RTB cDNAs (encoding wild-type or mutant RTB proceeded by the preproricin N-terminal signal sequence) in the vector pSP64T were transcribed in vitro in the presence of the capping dinucleotide 7-Me(5′)GpppG(5′)OH and SP6 polymerase as described earlier [7]. Purified RNA was dissolved in distilled water at a concentration of 1 mg/ml. Microinjection into *Xenopus laevis* oocytes (30 nl of RNA solution/oocyte) was performed using the Narashige microinjection system IM200. Pulse labeling with [^{35}S]methionine and oocyte homogenisation were performed as described previously [7].

2.3. Binding to immobilised lactose

The binding of biologically active RTB to lactose was determined as described previously [8,9]. Briefly, 1 ml of oocyte homogenate was passed down a small column containing 1 ml of lactose immobilised onto agarose (Pierce Chemical Co.). Unbound material was washed through the column, and bound material was eluted with 50 mM lactose. [^{35}S]RTB was recovered from collected fractions by immunoprecipitation using polyclonal rabbit anti-RTB antibodies, and its radioactivity determined by scintillation counting.

2.4. Binding to immobilised asialofetuin

Asialofetuin, 300 μl of 100 μg/ml solution in 0.1 M $NaHCO_3$, was dispensed into each well of a 96-well microtitre plate. After storing at 4°C overnight, unbound asialofetuin was removed and replaced with 300 μl of blocking solution (PBS, 0.5% (w/v) bovine serum albumin, 0.05% (v/v) Tween-20). After removing the blocking solution, a 200 μl sample of oocyte homogenate was added to each well and the plate was incubated at room temperature for 1 h. The sample was removed and the wells washed 3 times with PBS. Anti-RTB antibodies that we had raised in sheep (200 μl), diluted 1:100 in blocking solution, were added to each well. After 1 h at room temperature, the primary antibody solution was removed and the wells washed 3 times with PBS. Donkey anti-sheep IgG-alkaline phosphatase conjugate (Sigma) (200 μl), diluted 1:7500 in blocking solution, was added to each well. After 1 h at room temperature, the solution was removed and the wells washed 3 times with PBS. A 5 mg tablet of the alkaline phosphatase substrate, *p*-nitrophenyl phosphate, was dissolved in 5 ml of reaction buffer (9.7% (v/v) diethanolamine, 0.02% (w/v) sodium azide, 0.01% (w/v) $MgCl_2 \cdot 6H_2O$) immediately prior to use. A 200 μl volume of substrate solution was added to each well and the colour reaction monitored by measuring the absorbance at 405 nm. The reaction was stopped by the addition of 100 μl of 3 M NaOH per well. A calibration curve was prepared using known amounts of RTB in non-injected oocyte homogenate.

2.5. Other methods

Published procedures were followed for translation of the in vitro generated transcripts in a wheat-germ cell-free lysate [10], immunoprecipitation of RTB from oocyte homogenates [9], SDS-polyacrylamide gel electrophoresis and fluorography [11] and enzymic deglycosylation using endo H [12].

PII S0014-5793(97)00341-4

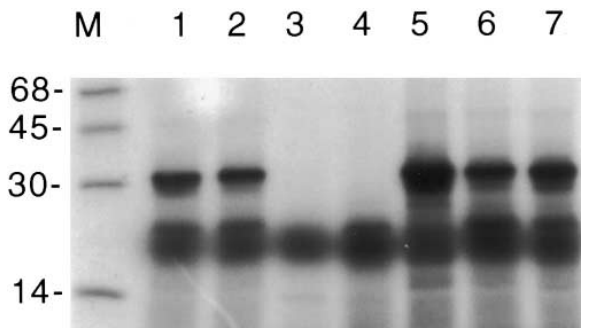

Fig. 1. Translation of RTB transcripts in a wheat-germ cell-free system in the presence of [^{35}S]methionine. Products were separated by SDS-PAGE and visualised by fluorography. Lane M, molecular mass markers (68, 45, 30 and 14 kDA as indicated); lane 1, Asn^{123}; lane 2, Asn^{42}; lanes 3 and 4, no added transcript; lane 5, RTB; lane 6, Asn^{42}/Asn^{123}; lane 7, BM5.

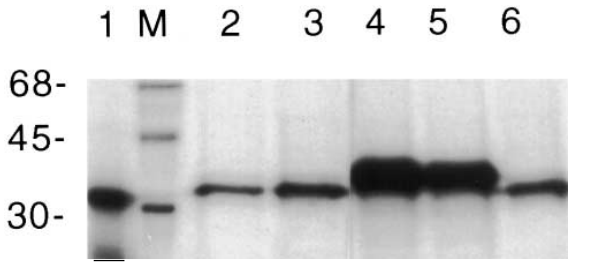

Fig. 2. Translation of RTB transcripts in *Xenopus* oocytes. Transcripts were injected into oocytes in the presence of [^{35}S]methionine. RTB was recovered from oocyte homogenates by immunoprecipitation, separated by SDS-PAGE, and visualised by fluorography. Lane 1, BM5; lane M, molecular mass markers; lane 2, Asn^{123}; lane 3, Asn^{42}; lane 4, RTB; lane 5, Asn^{42}/Asn^{123}; lane 6, Asn^{42}.

3. Results and discussion

The two native sugar attachment sites in RTB are Asn^{95} and Asn^{135}. The starting point for the present study was an RTB mutant (designated BM5) in which these two Asn residues had been replaced with Gln [8]. An examination of the primary sequence and the X-ray crystallographic structure of RTB revealed the presence of two Asn residues which are physically close to the original N-glycosylation sites in the 3-dimensional structure: Asn^{123} (which is close to Asn^{95}) and Asn^{42} (close to Asn^{135}). The position of Asn^{123} and Asn^{42} in the overall RTB structure also indicated that they might be able to accommodate N-linked oligosaccharides without significantly disrupting folding of the protein. Accordingly we used oligonucleotide site-directed mutagenesis to convert Asn^{123} and Asn^{42} into putative oligosaccharide attachment sites by changing Tyr^{125} and Asp^{44} respectively into Thr. The novel glycosylation sites were introduced into RTB either individually or in combination. The RTB mutants used in this study are listed in Table 1.

The RTB sequences were cloned into the transcription vector pSP64T, and in vitro transcripts were prepared for wild-type or mutant pre-RTBs using SP6 RNA polymerase [8]. Fig. 1 shows the products formed when the pre-RTB transcripts were translated in a wheat-germ cell-free system. A single major product of predicted molecular mass (~32 kDA) was synthesised in each case. Pre-RTB transcripts for proteins containing one or two N-glycosylation sites were microinjected into *Xenopus* oocytes and RTB was subsequently recovered from oocyte homogenates by immunoprecipitation using rabbit anti-RTB antibodies (Fig. 2). The oocyte products had higher apparent molecular masses than the wheat-germ products. Previous work has shown that pre-RTB transcripts injected to *Xenopus* oocyte generate product that is efficiently segregated into the oocyte ER, core-glycosylated, processed to remove the N-terminal signal peptide, and folded into the biologically active conformation stabilised by four intermolecular disulfide bonds [7]. The oocyte products contained either one or two oligosaccharide side chains as expected, and treatment with endo H to remove the attached glycans converted all the products to an apparent molecular mass of ~30 kDa, the predicted size of signal sequence cleaved, non-glycosylated RTB (Fig. 3).

The RTB variants were assayed for lectin activity. Oocyte homogenates containing radiolabeled RTBs were passed through a small column containing immobilised lactose. After washing the column to remove unbound material, bound lectin was eluted with 50 mM galactose and was recovered from the collected fractions by immunoprecipitation. As shown in Fig. 4, wild-type RTB had lectin activity whereas non-glycosylated BM5 was completely devoid of activity. The RTB mutant with a single novel N-glycosylation site at Asn^{123} (RTB Y125T) remained inactive, but the novel glycosylation site at Asn^{42} (RTB D44T) restored lectin activity. Mutant RTB containing both novel glycosylation sites (RTB D44T/Y125T) was inactive. RTB was the only protein eluted by galactose, and no galactose-eluted material was seen when radiolabeled homogenate from oocytes mock injected with water was applied to the column (data not shown). While this simple binding assay provides a convenient demonstration of lectin activity, only a small proportion of the immunoprecipitated RTB bound to the column (~11% for wild-type RTB and ~8% for the D44T glycosylated mutant). We have previously observed similar low levels of binding to immobilised lactose [8,9]. When the unbound RTB which passed directly through the column was reapplied to a second column, further RTB binding occurred which was similar in amount to that bound in the first affinity step (data not shown). Binding to the glycoprotein asialofetuin, which contains terminal galactose residues, is much more efficient. Asialofetuin is not a useful affinity matrix, however, since bound

Table 1
RTB N-glycosylation mutants used in the present study (amino acid single-letter code)

Mutations	Designation	Glycosylation sites
None	RTB	N95/G96/T97;N135/N136/T137
N95Q; N135Q	BM5	none
N95Q; N135Q; Y125T	Y125T	N123/I124/T125
N95Q; N135Q;D44T	D44T	N42/T43/T44
N95Q; N135Q; Y125T; D44T	D44T/Y125T	N123/I124/T125; N42/T43/T44

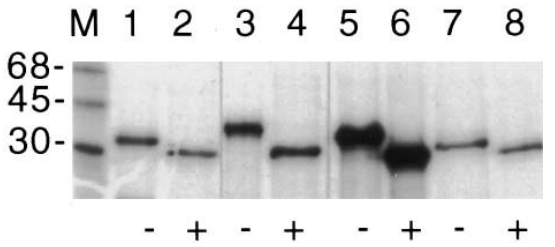

Fig. 3. Treatment of recombinant RTB with endo H. RTB transcripts were injected into *Xenopus* oocytes in the presence of [^{35}S]methionine and recovered by immunoprecipitation. Lanes labeled + and − represent endo H-treated samples and untreated controls, respectively. Lane M, molecular mass markers; lanes 1 and 2, Asn^{123}; lanes 3 and 4, Asn^{42}/Asn^{123}; lanes 5 and 6, RTB; lanes 7 and 8, Asn^{42}.

RTB cannot be displaced by solutions of free sugars. Accordingly, the lectin activity of the mutant RTB D44T with a glycosylation site at Asn^{42} was also demonstrated using an ELISA which monitored binding to immobilised asialofetuin as described in Section 2. The data, which are shown in Fig. 5, confirm the results from the immobilised lactose binding assay (Fig. 4). From a calibration curve prepared using known amounts of RTB in non-injected oocyte homogenate, the A_{405} values shown in Fig. 5 correspond to RTB concentrations of 62.5 ng/ml in homogenates from oocytes expressing wild-type RTB, and 50 ng/ml in homogenates from oocytes expressing the D44T glycosylation mutant, assuming both have equivalent lectin activity. While we have not attempted to accurately quantify the lectin activity of functional RTB variant, the results are qualitatively very clear: the glycosylation defective mutant is devoid of lectin activity, as is RTB Y125T with an introduced glycosylation site at Asn^{123}, or the

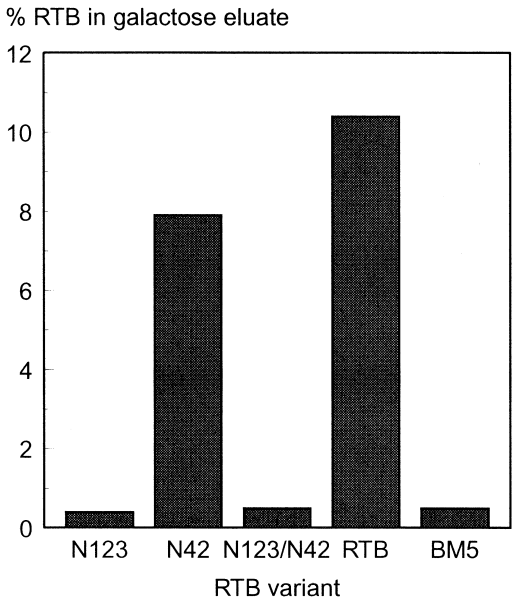

Fig. 4. Binding of recombinant RTB to immobilised lactose. Homogenates from oocytes expressing the appropriate RTB variant were applied to columns of immobilised lactose. The columns were washed with homogenisation buffer to remove unbound material (not shown) and then with buffer containing 50 nM galactose (shown). [^{35}S]RTB was recovered by immunoprecipitation from collected fractions (1 ml) and counted. The Fig. shows the RTB eluted by galactose as a % of total RTB in the applied sample.

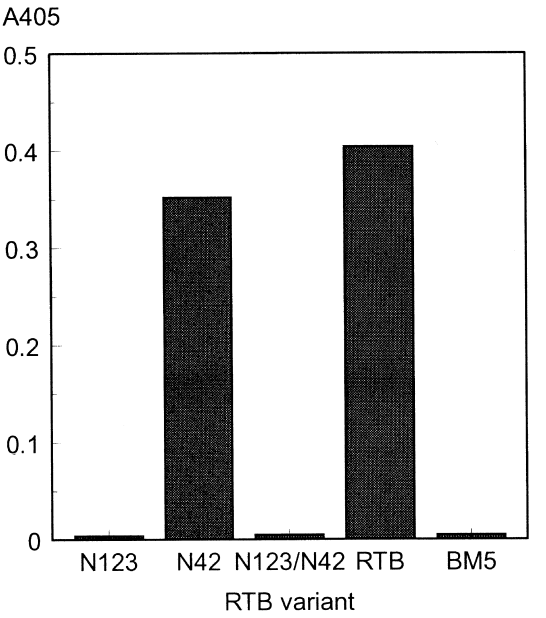

Fig. 5. Binding of recombinant RTB to immobilised asialofetuin. Bound RTB was determined by a sandwich ELISA using sheep anti-RTB antibodies followed by donkey anti-sheep antibodies conjugated to alkaline phosphatase as described in Section 2. Binding was indicated by alkaline phosphatase activity as an absorbance, corrected for background, at 405 nm.

mutant RTB D44T/Y125T doubly glycosylated at Asn^{42} and Asn^{123}, whereas RTB D44T with the single novel glycosylation site at Asn^{42} has lectin activity. Further the restored lectin activity of RTB D44T is of the same order as that determined for wild-type RTB. Clearly the lectin activity restored by the oligosaccharide at Asn^{42} is compromised when Asn^{123} is simultaneously glycosylated.

Why is N-glycosylation required for the lectin activity of recombinant RTB? We have previously found that non-glycosylated recombinant RTB produced in *Xenopus* oocytes is very unstable and rapidly aggregates on storage [8]. Likewise recombinant RTB produced in *Escherichia coli*, although initially soluble and biologically active, was unstable and tended to aggregate rapidly [13]. Clearly the oligosaccharide side chains of RTB appear to stabilise the native conformation. This observation, therefore, appears to be another example of the well established role for N-linked oligosaccharides in the folding and stability of glycoproteins (see [14] for review). The X-ray crystallographic structure of RTB shows that the two oligosaccharide side chains protrude into solution from close to that part of the polypeptide linking domains 1 and 2 [5]. It is possible that their strategic location in the RTB molecule somehow prevents unproductive interactions between domains 1 and 2 which lead to aggregation when the oligosaccharides are not present. Domains 1 and 2 of RTB have been expressed individually and remain soluble and biologically active whether they are [15] or are not [16,17] N-glycosylated. Thus it appears that in the absence of the second domain, domain 1 or domain 2 on their own do not require the protection of structural integrity apparently conferred on the bilobal RTB by the oligosaccharide side chains. In a molecule containing both domains but with the native N-glycosylation sites removed, the novel N-glycosylation site introduced at

Asn[42] also appears to introduce a stabilising oligosaccharide into mutant RTB which results in the retention of lectin activity.

Acknowledgements: This work was supported by the UK Biotechnology and Biological Sciences Research Council Grant 88/T 02035 and a Royal Society K.C. Wong Fellowship to J.Z. We thank Dr. J.D. Robertus (University of Texas at Austin) for advising us on the structural consequences of introducing novel glycosylation sites into RTB.

References

[1] J.M. Lord, L.M. Roberts, J.D. Robertus, FASEB J 8 (1994) 201–208.
[2] F.I. Lamb, L.M. Roberts, J.M. Lord, Eur J Biochem 148 (1985) 265–270.
[3] W. Montfort, J.E. Villafrance, A.F. Monzingo, S. Ernst, B. Katzin, E. Rutenber, N.H. Xuong, R. Hamlin, J.D. Robertus, J Biol Chem 262 (1987) 5398–5403.
[4] E. Rutenber, M. Ready, J.D. Robertus, Nature 326 (1987) 624–626.
[5] E. Rutenber, J.D. Robertus, Proteins 10 (1991) 260–269.
[6] A.E. Frankel, C. Burbage, T. Fu, E. Tagge, J. Chandler, M.C. Willingham, Biochemistry 35 (1996) 14749–14756.
[7] P.T. Richardson, P. Gilmartin, A. Colman, L.M. Roberts, J.M. Lord, Bio/Technology 6 (1988) 565–570.
[8] R. Wales, P.T. Richardson, L.M. Roberts, H.R. Woodland, J.M. Lord, J Biol Chem 266 (1991) 19172–19179.
[9] N. Sphyris, J.M. Lord, R. Wales, L.M. Roberts, J Biol Chem 270 (1995) 20292–20297.
[10] C.W. Anderson, J.W. Straus, B.S. Dudock, Methods Enzymol 101 (1983) 635–644.
[11] L.M. Roberts, J.M. Lord, Eur J Biochem 119 (1981) 31–41.
[12] J.M. Lord, Eur J Biochem 146 (1985) 411–416.
[13] K. Hussain, C. Bowler, L.M. Roberts, J.M. Lord, FEBS Lett 244 (1989) 383–387.
[14] H. Lis, N. Sharon, Eur J Biochem 218 (1993) 1–27.
[15] R. Wales, P.T. Richardson, L.M. Roberts, J.M. Lord, Arch Biochim Biophys 249 (1992) 291–296.
[16] C. Swimmer, S.M. Lehar, J. McCafferty, D.J. Chiswell, W. Blattler, B.C. Guild, Proc Natl Acad Sci USA 89 (1992) 3756–3760.
[17] R. Wales, H.C. Gorham, K. Hussain, L.M. Roberts, J.M. Lord, Glycoconjugate J 11 (1994) 274–281.