Double-site ricin B chain mutants retain galactose binding

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Three distinct double-site and two single-site ricin B chain (RTB) mutants were expressed in Spodoptera frugiperda insect cells and purified from infected cell supernatants. The yields of recombinant proteins were 0.01-0.2 mg/L. The purity after monoclonal antibody affinity chromatography was 1-20%. The mutant proteins were soluble, immuno-reactive with monoclonal antibodies and polyclonal antibodies to RTB and demonstrated molecular weights of 32 kDa, similar to plant RTB. All three double-site and both single-site mutants bound asialofetuin and mammalian cell surfaces based on an asialofetuin ELISA and cell binding immunofluorescence assay. While one double-site mutant, W37S/Y248S, had a 1 log drop in sugar binding, the other two double-site mutants W37S/Y248H and D22E/D234E had 2 log reductions in sugar binding. Each mutant reassOCIated efficiently (25-75%) with plant ricin A chain (RTA) to form cytotoxic heterodimers. The concentration of protein required to reduce protein synthesis 50% (ID₉₀) was 1 log higher than plant ricin for W37S/Y248S-RTA and the single-site mutant heterodimers, Q35N-RTA and D22E-RTA and 2 logs higher than plant ricin for the other two double-site mutant heterodimers. The results suggest amino acid residues in both the 1α and 2γ subdomains of RTB participate in sugar binding. However, other subdomains must contribute to the avidity of ricin for cell surface oligosaccharides.

Keywords: galactose binding/lectin/ricin

Introduction

Ricin toxin, the 65 kDa heterodimeric glycoprotein produced in castor bean seeds, is one of the most toxic substances known to man (Olson et al., 1974). The protein consists of a galactose-specific RTB disulfide linked to an rRNA N-glycosidase RTA. The toxin acts by binding cell surface glycolipids and glycoproteins with β-D-galactopyranose moieties (Baenziger and Fiete, 1979). Cell-bound ricin must then be internalized for cytotoxicity. Reticulocytes which lack endocytosis were insensitive to ricin (Olson et al., 1974). Mammalian cells incubated at 4°C or with metabolic inhibitors lost endocytosis and were not killed by ricin, although ricin bound the cells (Sandvig and Olson, 1982). After endocytosis via coated and uncoated pits, ricin must travel to the trans-Golgi network based on immunoelectron microscopy (van Deurs et al., 1986) and resistance of anti-RTB antibody producing hybridoma cells to ricin (Youle and Colombatti, 1987). After sorting in the Golgi, ricin undergoes transport to a distal compartment prior to translocation to the cytosol. Brefeldin A interrupts Golgi to endoplasmic reticulum (ER) vesicle transport and blocks ricin toxicity (Sandvig et al., 1991). Addition of the KDEL ER retention signal to RTA facilitates intoxication suggesting the ER is the critical compartment for membrane translocation to the cytosol (Wales et al., 1993). Membrane translocation is then triggered by interchain disulfide bond reduction followed by RTA unfolding. These steps have been blocked by thioether linkage between the ligand moiety and RTA (Masuho et al., 1982) or by stabilization of RTA tertiary structure with the introduction of a disulfide bridge (Argent et al., 1994). Finally, cytosolic RTA inactivates 60S ribosomal subunits by depurinating a critical adenine base in a highly conserved rRNA stem-loop (Endo et al., 1987). A single molecule of ricin introduced into the cytosol can produce cell death (Eiklid et al., 1980).

Immunotoxins, consisting of monoclonal antibodies covalently linked to peptide toxins, are a new class of therapeutics for cancer and autoimmune diseases (Frankel, 1993). The normal cell binding domains of the toxin must be modified to prevent unacceptable toxicities. Ricin-based immunotoxins have employed either RTA alone (Blythman et al., 1981) or blocked ricin in which glycopeptide cross-linkers have been used to modify lectin binding sites (Lambert et al., 1991a). RTA immunotoxin conjugates, including anti-CD5-RTA, anti-CD7-RTA, anti-CD25-RTA, anti-CD19-RTA and anti-CD22-RTA, anti-proteoglycan-RTA, anti-gp72-RTA, anti-transferrin receptor-RTA and anti-gp55-RTA have been administered to patients with refractory chronic lymphocytic leukemia and cutaneous T-cell lymphoma, acute lymphoblastic leukemia, Hodgkin’s disease, B-cell non-Hodgkin’s lymphoma, melanoma, colorectal carcinoma, peritoneal and meningeval carcinomatosis and breast carcinoma respectively (Frankel et al., 1995a). Only moderate clinical activity was seen in patients with hematopoietic malignancies which may be attributed in part to the poor delivery of toxin to translocation-competent vesicles of target cells (Oosterhout et al., 1992). Blocked ricin immunotoxin conjugates, including anti-CD19-blocked ricin and anti-CD56-blocked ricin, have been infused in patients with refractory B-cell non-Hodgkin’s lymphoma and small cell lung carcinoma respectively (Grossbard et al., 1993; Lynch et al., 1995). Dose-limiting toxicities to liver, megakaryocytes and the vascular endothelium were encountered suggesting residual normal tissue binding. These clinical results underscore the importance of an accurate analysis of the number and function of the lectin binding sites of ricin.

Equilibrium dialysis measurements with ricin showed affinities for galactose and lactose of approximately 10⁶/M (Zentz et al., 1978; Houston and Dooley, 1982), while cell binding experiments showed association constants for complex oligosaccharides and cell surfaces which were 3-4 logs higher (Sandvig et al., 1976; Baenziger and Fiete, 1979). These results suggest that multiple low affinity sugar binding sites on ricin interact with complex oligosaccharides and cells to yield high affinity binding.
Initial biochemical, crystallographic and mutational analysis suggested two ricin lectin pockets in subdomains 1α and 2γ. N-Bromosuccinimide modification of Trp37 reduced sugar binding, demonstrating a sugar binding site in subdomain 1α (Hatakeyama et al., 1986). N-Acetyl-\textit{d}{-}acetylation of Tyr248 reduced sugar binding implicating a binding site in subdomain 2γ (Yamasaki et al., 1985). X-ray diffraction analysis to 2.5 Å resolution of ricin crystals soaked in 5 mM α-lactose revealed that lactose molecules bound to amino acid side chains in the 1α and 2γ subdomains. Mutant recombinant RTB molecules were expressed, partially purified and characterized in Xenopus laevis oocytes, Cos mammalian cells and as fusion proteins on bacteriophage and binding to immobilized asialofetuin assayed (Vitetta and Yen, 1990; Wales et al., 1991; Swimmer et al., 1992). In each case, modifications of amino acid residues in either one (Vitetta and Yen, 1990) or two subdomains (Wales et al., 1991; Swimmer et al., 1992) ablated lectin activity.

However, a review of data from clinical trials, cell cytotoxicity studies, biochemical modification work, X-ray crystallography and analyses of partially purified mutant RTBs leads to a hypothesis that ricin has three lectin sites. Patients treated with a triply blocked ricin conjugate had none of the toxicities observed with a doubly blocked ricin conjugate (Grossbard et al., 1992). In vitro, the residual non-specific cytotoxicity of the doubly blocked ricin conjugates was blocked with lactose. Further, cytotoxicity to antigen-bearing cells was observed with antibody conjugated to ricin with two cross-linked ligands but not three ligands, even in the presence of lactose. Thus, the third sugar-combining site appeared necessary to facilitate conjugate intoxication. Treatment of ricin with N-acetyl-\textit{d}{-}acetylation of a second tyrosine with further loss of galactose binding. These results suggest two tyrosine containing lectin subdomains, in addition to the tryptophan containing lectin site (Youle et al., 1981). Exposure of ricin to radiolabeled fetuin glycopeptide containing a dichlorotriazine-activated 6-(N-methylamino)-6-deoxy-\textit{d}{-}galactose moiety produced up to three cross-linked peptides per ricin. Lectin activity was inversely related to the number of bound ligands (Lambert et al., 1991). In vitro, X-ray crystallography of ricin showed six homologous subdomains with similar folding and primary amino acid sequence. The subdomains resembled the primitive galactose binding fold in discoidin I. Three of these subdomains (1α, 1β and 2γ) had tripeptide kinks in the \( \alpha \)-carbon chain forming lactose binding pockets and had aromatic ring residues for hydrophobic interaction with the non-polar face of galactose (Rutenberg and Robertus, 1991). In previous studies, our laboratory has expressed, partially purified and characterized wild type and single-site RTB mutants in insect cells (Frankel et al., 1996). Wild type recombinant RTB bound asialofetuin and cell surface oligosaccharides similar to plant RTB with half-maximal binding concentrations of \( \sim 5 \times 10^{-8} \text{ M} \). Single-site mutants of both the 1α subdomain (K40M, Q35N, W37S and N44G/K40M) and the 2γ subdomain (N255G, N255A, Y248S and D234E/A237R) had reduced binding affinity for asialofetuin and KB mammalian cells. However, the reduction in binding affinity was 1 log or less in each case. After reassociation with plant RTA, each single-site mutant retained HUT102 human leukemia cell cytotoxicity with ID\(_{50}^{\text{A}}\) within 1–1.5 logs of the wild type heterodimer. The minor effect of genetic modification of lectin sites in subdomains 1α and 2γ suggests incomplete inactivation of lectin sites or additional sugar-combining sites on RTB. We now describe the expression, partial purification and characterization of three double-site mutant RTBs and two single-site mutant RTBs. Two of the double-site mutants had unique properties supporting either the persistence of two main galactose binding sites operating at reduced levels or the theory of three ricin lectin sites.

### Materials and methods

Restriction endonucleases and T4 ligase were obtained from Promega (Madison, WI, USA). Isotopes and the Sculptor in vitro mutagenesis system were obtained from Amersham (Arlington Heights, IL, USA). Polyclonal antibodies and chemicals were from Sigma (St Louis, MO, USA). EX-CELL 400 medium was obtained from JRH Scientific (Lexena, KS, USA). S9 insect cells, TMNFH medium, BaculoGold DNA and pAcGP67A transfer vector were from PharMingen (San Diego, CA, USA). DNA and plasmid prep kits were obtained from BioRad (Hercules, CA, USA). The Sequenase kit for dideoxy sequencing was obtained from USB (Cleveland, OH, USA). The Random Primer labeling kit and M13K07 phage were obtained from Stratagene (La Jolla, CA, USA). Purified P2, P8 and P10 murine monoclonal antibodies to RTB and purified αBR12 murine monoclonal antibody to RTA were gifts of Dr Walter Blattler, ImmunoGen (Cambridge, MA, USA). Sera and media were obtained from Gibco BRL (Grand Island, NY, USA). 3M Emphase Biosupport medium and binding to immobilized asialofetuin assayed (Vitetta and Yen, 1990; Wales et al., 1991; Swimmer et al., 1992) ablated lectin activity.

#### Construction of transfer vectors encoding mutant RTBs

pUC119-RTB plasmid containing a \textit{BamHI}–\textit{EcoRI} DNA fragment coding for ADP-RTB was propagated in INVαF' cells as previously described (Frankel et al., 1994). Single-stranded DNA was produced by infection of transformants with M13K07 phage as previously described (Schlossman et al., 1989). Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and desalted with butan-1-ol. Thirty-nine-mers were prepared with the modified codon flanked by 18 bases on each side matching the RTB sequence and lacking an Ncol site. Site-specific mutagenesis was performed by the Eckstein method using the Amersham in vitro mutagenesis kit and manufacturer's instructions (Olson and Eckstein, 1990). Modifications were made at both the 1α and 2γ subdomains based on the X-ray crystallographic model of the lectin binding sites (Figure 1) to either alter key polar residues which provide hydrogen bonds to sugar hydroxyls (D22E/D234E, Q35N and D22E) or change aromatic ring residues which provide van der Waals interactions between the protein and sugar (W37S/Y248S and W37S/Y248H). The sequences of mutant RTB DNAs were confirmed by double-stranded dideoxy sequencing by the Sanger method using the Sequenase kit (Sanger et al., 1977). Mutant RTB encoding pUC119 DNAs were then restricted with \textit{BamHI} and \textit{EcoRI} and the RTB encoding fragments were subcloned into pAcGP67A plasmid and used to transform INVαF' cells. Transfer vectors with mutant RTBs were then purified by cesium chloride gradient centrifugation.

#### Isolation of recombinant baculoviruses

The Sf9 \textit{Spodoptera frugiperda} ovarielle cell line was maintained on TMNFH medium supplemented with 10% fetal calf serum.
pAcGP67A-mutant RTB DNAs (4 µg) were co-transfected with 0.5 µg of BaculoGold AcNPV DNA into 2 × 10⁶ Sf9 insect cells following the recommendations of the supplier. On day 7 post-transfection, media were centrifuged and the supernatants tested in limiting dilution assays with Sf9 cells. The 2 × 10⁶ Sf9 cells were incubated with 10-fold dilutions of supernatants in 96-well plates. Seven days post-infection, supernatants were saved and cells in each assay well were lysed with NaOH and the lysates transferred to nitrocellulose. The nitrocellulose was then blocked with Blotto and reacted with random primer ³²PdCTP-labeled RTB DNA. After hybridization for 16 h at 67°C, the dot blot membranes were washed with 0.1 X (150 mM NaCl/15 mM sodium citrate)/1% SDS, dried and exposed to X-ray film. Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10⁻⁷ dilution were positive. Two rounds of selection were required for each mutant. Recombinant viruses in the supernatants were then amplified by infecting Sf9 cells at an m.o.i. of 0.1, followed by collection of day 7 supernatants.

Expression of mutant B chains in Sf9 cells
Recombinant baculoviruses were used to infect 2 × 10⁸ Sf9 cells at an m.o.i. of 5 in EX-CELL 400 media with 50 mM α-lactose in spinner flasks. Media supernatants containing mutant RTBs were collected day 6 post-infection.

Purification of mutant RTBs
Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4°C. The supernatants were concentrated 15-fold by vacuum dialysis, centrifuged at 3000 g for 10 min to remove precipitate, dialyzed against 50 mM NaCl, 25 mM Tris, pH 8, 1 mM EDTA, 0.01% sodium azide and 25 mM α-lactose (NTEAL), ultracentrifuged at 100 000 g for 1 h and loaded onto a P2 monoclonal antibody-acylamide column as previously described (Frankel et al., 1994). The affinity column was then washed sequentially with NTEAL and 500 mM NaCl, 25 mM Tris, pH 9, 1 mM EDTA, 0.01% sodium azide, 25 mM α-lactose and 0.1% Tween-20 (NTEALT) and mutant RTBs eluted with 0.1 M triethylamine, pH 11. The alkaline eluants were immediately neutralized with 1 M sodium phosphate, pH 4.8, and stored at -20°C until assayed. The optical densities at 280 nm were determined and aliquots mixed with reducing 2 X SDS sample buffer, boiled for 4 min, electrophoresed on a 15% SDS-PAGE, stained with Coomassie blue R-250 and destained with acetic acid/methanol. Gels were scanned on an IBAS automatic image analysis system to estimate the fraction of protein of molecular weight 32 kDa.
**Immunological properties of mutant RTBs**

Aliquots of mutant RTBs, plant RTB, wild type recombinant RTB and pre-stained low molecular weight standards were mixed with reducing 2 × SDS sample buffer, boiled for 5 min and loaded on a 15% SDS–PAGE, and electrophoresed for 90 min. Gels, Whatman 3M no. 1 paper and nitrocellulose were equilibrated for 15 min in Towbin buffer (20 mM Tris/0.1 M glycine/20% methanol) and placed in a Semi-dry Transblot cell (BioRad). After electrophoresis at 15 V for 20 min, the nitrocellulose was blocked with 10% Carnation’s non-fat dry milk/0.1% BSA/0.1% Tween-20/0.02% sodium azide. The blots were then washed with PBS plus 0.05% Tween-20 and PBS, incubated with rabbit anti-ricin antibody at 1:400 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) at 1:1000 in PBS plus 0.5% BSA plus 0.01% sodium azide for 1 h, washed again and developed with the Vectastain alkaline phosphatase kit, following the manufacturer’s recommendations. Blots were scanned as above to compare 32 kDa Mr band intensities.

Monoclonal antibody P2, P8 or P10 (100 μl) at 5 μg/ml in PBS was incubated in Costar EIA microtiter wells overnight at 4°C. Samples of plant RTB, wild type recombinant RTB and mutant RTBs were treated for 20 min at room temperature with 5% β-mercaptoethanol and then dilutions made in EX-CELL 400. The antibody-coated microtiter wells were then washed with PBS plus 0.1% Tween-20, blocked with 3% BSA/PBS/0.01% sodium azide, rewashed and incubated with dilutions of the reduced RTB samples, rewashed and incubated with rabbit anti-ricin antibody 1:400 in PBS plus 0.5% BSA plus 0.01% sodium azide, washed again, incubated with alkaline phosphatase conjugated goat anti-(rabbit IgG) at 1:1000 in PBS plus 0.5% BSA plus 0.01% sodium azide, washed and developed with p-nitrophenylphosphate at 1 mg/ml in 50 mM diethanolamine buffer, pH 9.8, and read on a BioRad 450 Microplate reader at 405 nm. For each experiment 12 different concentrations of plant RTB and recombinant RTBs were tested. A plot of absorbance versus dilution was made for plant RTB and recombinant proteins. Dilutions yielding half-maximal binding were used to calculate concentrations.

**Lectin activity of mutant RTBs**

One hundred microliter volumes of 1 μg/ml asialofetuin in PBS were added to wells of a Costar EIA plate and incubated overnight at 4°C. Samples of plant RTB, wild type recombinant RTB or mutant RTBs in EX-CELL 400 were exposed to 5% β-mercaptoethanol for 20 min at room temperature to remove homodimers and dilutions made in EX-CELL 400 medium with or without 20 μg/ml asialofetuin or 100 mM α-lactose. The asialofetuin-coated microtiter wells were then washed with PBS/0.1% Tween-20, blocked with 3% BSA/PBS/0.01% sodium azide and rewashed. The dilutions of various reduced RTBs were added to wells for 1 h and then removed and the wells washed again. Rabbit anti-ricin antibody was added (1:400 dilution in 0.5% BSA/PBS/0.1% sodium azide) for 1 h, the wells were washed again, alkaline phosphatase conjugated goat anti-(rabbit IgG) (1:5000 in 0.5% BSA/PBS/0.01% sodium azide) incubated in the wells and, finally, the wells were washed and reacted with 1 mg/ml p-nitrophenylphosphate in 50 mM diethanolamine buffer, pH 9.6, and measured in a microtiter plate reader at 405 nm. In each experiment, 12 different concentrations of plant RTB and recombinant protein were tested. As in the antibody ELISA, the relative reactivity to plant RTB was calculated from concentrations giving half-maximal binding. The effects of 100 μg/ml asialofetuin or 100 mM α-lactose on half-maximal binding were calculated for plant RTB, wild type recombinant RTB and mutant RTBs.

KB cells were washed with PBS and attached to polylysine-coated tissue-culture dishes and centrifuged at 2000 g for 10 min. The cells were then incubated live at 4°C. The cells were washed with 2 mg/ml BSA in PBS and incubated in PBS plus BSA with or without 100 μg/ml asialofetuin and with 1 μg/ml freshly reduced plant RTB, recombinant wild type RTB or mutant RTB. The incubation was done at 4°C. The cells were then washed with PBS and incubated with rabbit anti-ricin antibody at 1:400 in PBS plus BSA for 30 min at 4°C. The cells were then washed with PBS and reacted with goat anti-(rabbit Ig) conjugated to rhodamine at 25 μg/ml for 30 min at 4°C. The cells were washed again in PBS and fixed in 3.7% formaldehyde in PBS, mounted under a no. 1 coverslip in glycerol–PBS (90:10) and examined under a Zeiss Axioplan epifluorescence microscope.

**Reassociation of mutant RTBs with plant RTA to form heterodimers**

Thirty microgram portions of plant RTB and wild type recombinant RTB and 1-5 μg mutant RTBs were mixed with a 3-fold molar excess of plant RTA in a total volume of 0.5 ml of 0.1 M triethylenemine-sodium phosphate, pH 8, with shaking overnight at room temperature. The reaction mixture was then analyzed by a modified ricin ELISA. Wells of an EIA plate were coated with 10 μg/ml P2 monoclonal antibody to RTB diluted in PBS in a volume of 100 μl overnight at 4°C. The wells were washed with PBS plus 0.1% Tween-20, blocked with 3% BSA in PBS plus 0.02% sodium azide, rewashed and incubated with dilutions of ricin or reassociated heterodimers. The wells were again washed and incubated with biotin conjugated αBR12 monoclonal antibody at 5 μg/ml in PBS/0.5% BSA/0.1% sodium azide. Biotinylation was performed using N-hydroxysuccinimidobiotin (Sigma) following the manufacturer’s instructions. Wells were washed and incubated with alkaline phosphatase conjugated streptavidin (Sigma) at 1:1000 in PBS/0.5% BSA/0.1% sodium azide, washed again and developed with p-nitrophenylphosphate at 1 mg/ml in 50 mM diethanolamine, pH 9.8. Absorbance at 405 nm was read on a microtiter plate reader. Reassociated mixtures were also analyzed by non-reducing SDS–PAGE followed by immunoblots with αBR12 anti-RTA monoclonal antibody and P10 anti-RTB monoclonal antibody. Densitometric scanning using the IBAS 2000 automatic image analysis system (Kontron, Germany) was done to quantify the shift of immunoreactive material from 30 to 60 kDa.

**Cytotoxicity of recombinant mutant heterodimers**

A 1.5 × 10^8 aliquot of HUT102 human T leukemia cells in 100 μl was placed in 96-well flat-bottomed plates in leucine-poor RPMI 1640 containing 10% dialyzed fetal bovine serum. Fifty microliters of ricin, recombinant wild type RTB–plant RTA heterodimer and mutant RTB–plant RTA heterodimers at varying concentrations were added in the same medium and the cells incubated at 37°C in 5% CO_2 for 24 h. [^3]H]Leucine, 0.5 μCi per well (120 μCi/mmol), in 50 μl of the same medium was added and incubated for 4 h. Cells were then harvested with a PhD Cell Harvester onto glass-fiber filter mats. The filters were dried, mixed with 3 ml of liquid scintillation fluid and counted in an LKB-Wallac liquid scintil-
lution counter gated for \(^3\text{H}\). Cells cultured with medium alone served as controls. All assays were performed in triplicate. In some experiments duplicate samples were incubated in the presence of 50 mM \(\alpha\)-lactose. The ID\(_{50}\) was the concentration of protein which inhibited protein synthesis by 50% compared with the control.

**Results**

**Yields and immunoreactivity of mutant RTBs**

Each of the three double-site mutants contained modifications in amino acid residues in both the 1\(\alpha\) and 2\(\gamma\) subdomains. None of the three mutants (D22E/D234E, W37S/Y248S and W37S/Y248H) have been previously described. One of the single-site mutants, Q35N, has been prepared previously (Frankel et al., 1996); the other single-site mutant, D22E, has not been previously described.

The yields were estimated from the optical density at 280 nm of neutralized alkaline eluants post-affinity chromatography (plant RTB OD = 1.44 for 1 mg/ml) and densitometry of Coomassie-stained reducing SDS-PAGE (10–30% of the protein migrated at 33 kDa; Figure 2A). These results were confirmed by densitometry of immunoblots with polyclonal rabbit anti-ricin antibody. As shown in Figure 2(B), all of the double-site mutants were reactive with the polyclonal antibody. Similar results were obtained with the single-site mutants. Finally, a monoclonal antibody anti-RTB ELISA was used to verify the concentrations of each mutant. All three assays gave similar values. Purified wild type RTB was obtained at 300–500 \(\mu\)g/ml culture fluid, while the mutants were recovered at 2–15-fold lower amounts. The yield of D22E/D234E was 23 \(\mu\)g/ml, the yield of W37S/Y248S was 22 \(\mu\)g/ml, the yield of W37S/Y248H was 140 \(\mu\)g/ml and the yield of a second independent isolate of W27S/Y248H was 270 \(\mu\)g/ml. The yield of D22E was 150 \(\mu\)g/ml and the yield of Q35N was 100 \(\mu\)g/ml. The lower yields of the D22E/D234E and W37S/Y248S mutants may be due to degradation of improperly folded proteins. Other investigators have reported bioactivity of mutants without purification or characterization (Vitetta and Yen, 1990; Wales et al., 1991). Their assumption that modification of lectin site residues would not affect secondary structure is perhaps inaccurate and overemphasizes the role of individual residues and subdomains in sugar binding and cytotoxicity.

<table>
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<th>Protein</th>
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<th>Relative binding to asialofetuin (%)</th>
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<td>Frankel et al. (1995b)</td>
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The reactivities of mutant RTBs with different monoclonal antibodies to RTB (P2, P8 and P10) were tested substituting different monoclonal antibodies as capture reagents in the...
Fig. 3. Binding of mutant RTBs to KB cells. Cells were attached to polylysine-coated tissue culture dishes and all incubations were done at 4°C. The cells were washed with 2 mg/ml BSA in PBS and the PBS plus BSA plus 1 μg/ml of purified mutant RTB or wild type RTB, rewarshed, incubated with 1:200 rabbit anti-ricin antibody (Sigma) in PBS plus BSA, rewarshed, incubated with affinity purified goat anti-(rabbit Ig) coupled to rhodamine (Jackson ImmunoResearch) at 25 μg/ml, washed again and fixed in 3.7% formaldehyde in PBS (Magnification = ×250, bar = 20 μm; arrows = surface fluorescence pattern.) (A, C, E and G) Without 100 μg/ml asialofetuin. (B, D, F and H) With asialofetuin. (A and B) Wild type RTB, (C and D) W37S/Y248H, (E and F) D22E/D234E, (G and H) W37S/Y248H no. 2.

Antibody ELISA. Equivalent results were observed for each antibody suggesting similar folding of all the mutants. The concentration of wild type recombinant RTB was estimated at 70 μg/ml for P2 antibody capture and 70 μg/ml for P8 and 10 μg/ml for P10 antibody capture. D22E/D234E concentrations were 3 μg/ml with P2, 2 μg/ml with P8 and 3 μg/ml with P10. W37S/Y248S gave 2 μg/ml with P2, 2 μg/ml with P8 and 5 μg/ml with P10. W37S/Y248H was 60 μg/ml with P2, 40 μg/ml with P8 and 25 μg/ml with P10. A second isolate of W27S/Y248H showed 10 μg/ml with P2, 10 μg/ml with P8 and 10 μg/ml with P10. D22E was 5 μg/ml with P2, P8 and P10.

Fig. 4. Reassociation of mutant RTBs with plant RTA. Immunoblots of 15% non-reducing SDS–PAGE of reassociated RTB-plant RTAs. Arrows indicate low molecular weight BioRad protein standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa). Heterodimer appears at 60 kDa and subunits at 30 kDa. (A) Immunoblot using monoclonal antibody P10 anti-RTB. (B) Immunoblot using monoclonal antibody dbR12 anti-RTA. Lane 1, low molecular weight prestained BioRad protein standards; lane 2, D22E/D234E; lane 3, W37S/Y248H no. 2; lane 4, W37S/Y248H no. 1; lane 5, W37S/Y248S.

Sugar binding of mutant RTBs

Binding of partially purified double-site mutant RTBs to immobilized asialofetuin was quantitated by ELISA and the results are shown in Table I. The single-site mutants and the double-site W37S/Y248S mutant showed less than a 1 log drop in binding relative to recombinant or plant RTB. The other two double-site mutants (D22E/D234E and W37S/Y248H) showed close to a 2 log drop in sugar binding. The limit of detection of the assay was a 2.5–3 log decrease in sugar binding avidity. In each case, the binding of mutants to immobilized asialofetuin could be blocked >10-fold with 100 mM α-lactose or 20 μg/ml asialofetuin.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. All the double-site mutants bound KB cells at 4°C (Figure 3A). Binding of wild type recombinant and all three mutants was blocked by asialofetuin (Figure 3B). The single-
of reassociated heterodimer tested by modified ELISA. Each experiment was quantitated by a modified ricin ELISA performed in triplicate. 

\[ \text{RTA ID}_{50} = 9 \times 10^{-12} \text{ M; } \] 
\[ \text{A, D22E/D234E-plant RTA ID}_{50} = 9 \times 10^{-11} \text{ M; } \] 
\[ \text{W37S/Y248S-plant RTA ID}_{50} = 1 \times 10^{-11} \text{ M; } \] 
\[ \Delta, \text{D22E/D234E-plant RTA ID}_{50} = 9 \times 10^{-11} \text{ M.} \]

Yield of reassociated heterodimer tested by modified ELISA. Each experiment performed in triplicate.

Site mutants, Q35N and D22E, bound KB similarly to previous studies with single-site mutants (Frankel et al., 1996).

**Competition experiments**

Binding of recombinant proteins to immobilized asialofetuin was performed in the presence of 100 mM α-lactose and 100 μg/ml asialofetuin. In each case, the sugar binding of the RTB protein was inhibited by competitor. Lactose blocked wild type RTB binding 27-fold and asialofetuin blocked wild type RTB binding 81-fold. W37S/Y248H was blocked 27-fold by lactose and 50-fold by asialofetuin. W37S/Y248H was blocked 9-fold by lactose and 15-fold by asialofetuin. D22E/D234E binding was inhibited 9-fold by lactose and 15-fold by asialofetuin. The inhibition by soluble carbohydrates of double-site mutant binding was a lower estimate as the assay sensitivity was only 5- to 10-fold less than the observed binding to immobilized asialofetuin in the absence of added sugars for two of the double-site mutants. Each experiment was repeated three times and 12 different concentrations of recombinant protein and plant RTB were used to compare half-maximal binding concentrations.

**Reassociation of mutant RTBs with plant RTA**

Incubation of mutant RTBs at \( 3 \times 10^{-8} \text{ M} - 3 \times 10^{-7} \text{ M} \) with plant RTA at a 3-fold molar excess overnight at room temperature yielded 65% reassociation of W27S/Y248H, 75% reassociation for a second preparation of W37S/Y248H, 50% reassociation of D22E/D234E and 25% reassociation of W37S/Y248S (Figure 4 and caption to Figure 5). Reassociation of single-site RTB mutants with plant RTA was also observed with 65% of Q35N coupling to plant RTA and 25% of D22E linking to plant RTA. Similar levels of reassociation were seen using plant RTB or recombinant wild type RTB with plant RTA at the same concentrations. The heterodimer concentrations were quantitated by a modified ricin ELISA which identifies molecules with both RTA and RTB epitopes and by densitometry of 64 kDa bands of immunoblots with anti-RTB or anti-RTA antibodies. Both ELISA and immunoblots gave similar values and showed all mutants reassociated well with plant RTA and had minimal homodimer formation.

**Cytotoxicity of mutant heterodimers**

The ID_{50} of ricin on HUT102 cells was \( 3 \times 10^{-12} \text{ M} \) (Figure 5). We found insect RTB–plant RTA had a comparable ID_{50} of \( 5 \times 10^{-12} \text{ M} \). The double-site mutant–plant RTA heterodimers showed higher ID_{50}s. W37S/Y248S–plant RTA had an ID_{50} of \( 1 \times 10^{-11} \text{ M} \). W37S/Y248H–plant RTA had an ID_{50} of \( 9 \times 10^{-11} \text{ M} \). D22E/D234E–plant RTA had an ID_{50} of \( 9 \times 10^{-11} \text{ M} \) and the second preparation of W37S/Y248H–plant RTA gave an ID_{50} of \( 3 \times 10^{-10} \text{ M} \). The reduction in potency was 3- to 5-fold for W37S/Y248S–RTA, 25-fold for W37S/Y248H–RTA and D22E/D234E–RTA and 100-fold for the second preparation of W37S/Y248H–RTA.

**Discussion**

The yields of double-site mutants varied 12-fold and were reproducibly lower than wild type RTB. Misfolding followed by proteolytic degradation may contribute to the lower yields of the mutants. The molecular mechanism for misfolding was examined in greater detail by studying the X-ray crystallographic structure of RTB (Rutenber and Robertus, 1991). Energetic contributions to the final RTB structure include Ω-loops connecting the core and solvent, core hydrophobic interactions, solubilization due to attached carbohydrates, interfacial interactions with RTA and lectin pocket bonding with galactose. The Ω-loops within each subdomain are compact, contiguous peptide segments with ‘loop-shaped’ paths in 3-D space. The Ω-loops are stabilized by sets of hydrogen bonds between the backbone nitrogen and carbonyl oxygen atoms. In addition, Ω-loops in the α and β subdomains have disulfide bonds securing the neck of the loop. The RTB core contains hydrophobic residues from each subdomain which have extensive van der Waals interactions. The contact area with RTA covers 14% of RTB and includes both hydrophobic interactions (F262, P260, F140 and F218) and polar interactions (A1, D2, D94, V141, K219 and N220). N-Linked oligosaccharides are attached to N95 and N135 in insect RTB (Frankel et al., 1994). Finally, interactions with carbohydrate include RTB lectin site polar residues and aromatic ring residues. All the amino acid residues critical to Ω-loop stabilization, core formation, N-linked glycosylation and interfacial interactions with RTA are distant from the lectin sites. In contrast, the mutants were specifically selected to reduce galactose bonding. The small number of bonds involved in sugar binding does not contribute large negative free energy to the final structure of RTB, but may aid in the stabilization of proper folding intermediates. In support of this hypothesis, refolding of denatured recombinant *Escherichia coli* RTB which lacks glycosylation and RTA was enhanced by the presence of lactose (Tonevitsky et al., 1994). Since the recombinant insect RTB molecules lack RTA during folding, the effect of the lost sugar ligand stabilization during folding may be crucial.

Predictions of the role of individual amino acid modifications on lectin site structure and function is dependent upon the maintenance of protein tertiary structure. Without sufficient quantities of mutants for crystal production and X-ray diffraction analysis, we used indirect methods to confirm proper folding. The mutants retained solubility and stability similar...
to plant RTB implying an energetically favorable conformation. Immunoreactivity with both polyclonal and monoclonal antibodies to RTB suggested preservation of epitopes. Efficient reassociation with RTA and potent cell cytotoxicity of heterodimers suggests intact functional domains. RTB mutant N255A produced in Cos cells was not characterized for solubility (Vitetta and Yen, 1990). RTB single- and double-site mutants produced in X. laevis oocytes were metabolically labeled, not purified and not characterized immunologically (Wales et al., 1991). Both groups of investigators assumed the amino acid modifications would not affect the secondary structure or yields of properly folded molecules. With partial purification and extensive immunobossays, we observed significant variation in yield that, if not recognized, would have given falsely low sugar binding affinities.

The higher binding activity of the W37S/Y248S mutant may be due to incomplete inactivation of the 2γ subdomain lectin site. In support of this hypothesis, the W37S/Y248H mutant reproducibly showed 1 log lower binding than the W37S/Y248S mutant. The hydropathic index is significantly different from histidine relative to serine or tyrosine and may contribute to greater structural perturbation by histidine (Kyte and Doolittle, 1982). Further, the histidine imidazole moiety is much larger than the serine hydroxyl and may limit adjustments in secondary and tertiary structure at the lectin site. The Y248H mutation occurs naturally in ricin agglutinin and ricin E and the affinity of both these molecules is reduced significantly for cells and galactosides (Araki and Funatsu, 1987). A gene 3 fusion protein with Y248H RTB on the surface of fd phage also showed reduced sugar binding (Lehar et al., 1994). There are no natural ricin variants with serine at position 248. The single-site mutant Y248S RTB expressed in insect cells had one-third the binding avidity of wild type RTB (Frankel et al., 1996). Since the avidity of Y248S was reduced to a similar degree as N255G, N255A and D234E/A237R, we did not expect to see incomplete lectin site inactivation by Y248S. The measurement of residual binding in the double-site mutants may more accurately show differences in avidity.

The results of cell binding immunofluorescence studies confirmed the observations made with immobilized asialofetuin. Thus, the mutant heterodimers retain at least some of the galactosyl binding functions of plant ricin. Since binding was assayed at lectin concentrations of 1 μg/ml corresponding to 3 × 10⁻⁷ M, at least moderate affinity interaction with cell surfaces was retained in the mutants. These results differ from the observations of absent sugar binding for the single-site RTB mutant expressed in Cos cells (Vitetta and Yen, 1990) and the double-site RTB mutants expressed in X. laevis oocytes (Wales et al., 1991). However, as noted above, no efforts were made to purify or characterize the Cos cell mutant or Xenopus mutants and the range of the binding assays was not detailed.

The implications of a third sugar combining site on ricin are broad. Most type II carbohydrate binding proteins including plant lectins have multiple shallow surface sugar binding grooves with low individual affinities ($K_a = 10^{-3}$–$10^{-7}$ M) (Vyas, 1991). High affinity binding is achieved by a cluster glycoside effect due to multiple lectin sites per molecule (Lee, 1992). The presence of a third lectin site on ricin coincides with observations on the hepatic Gal/GalNac lectin (Lee et al., 1987) and mannose-binding proteins (Weis and Drickamer, 1994).

A third lectin site on ricin provides a molecular mechanism for the residual lectin function of double-blocked ricin and double-blocked ricin antibody conjugates (Grossbard et al., 1992). The enhanced cytotoxicity of double-blocked ricin antibody conjugates compared to RTA conjugates can then be explained as secondary to residual galactose binding facilitating both internalization of immunotoxin and transfer to a translocation-competent vesicle (Goldmacher et al., 1992). The cytotoxicity of the double-site mutant heterodimers in the current study is further evidence for the capacity of the third lectin site to provide critical intoxication functions such as internalization and intracellular routing.

The toxicities observed in the blocked ricin clinical trials may be due to non-specific uptake of toxin by normal tissues; however, the disappearance of toxicities in patients receiving triply locked ricin conjugate argues for a role of the third lectin site in the dose-limiting toxicities of the current double-blocked ricin conjugates (Grossbard et al., 1992).

Finally, genetic engineering or ricin-based fusion proteins similar to Pseudomonas exotoxin fusion molecules or diphtheria toxin hybrids (Pastan and FitzGerald, 1992) will require ablation of the normal tissue binding sites. The current study suggests at least three lectin sites will need to be modified to provide a large enough therapeutic window.

Blocking of mutant RTB binding to immobilized asialofetuin or KB cells by lactose or soluble asialofetuin confirms the specificity of the reaction and argues against non-specific interactions with surfaces as the source of the residual binding. Further, wells coated with bovine serum albumin alone show no binding in the asialofetuin assay to mutants or wild type recombinant RTB. Extending these observations, peptide analysis of affinity cross-linked ricin molecules (Lambert et al., 1991b) should permit definition of the third lectin site.

Reassociation functions in the double-site mutant RTBs appear intact. As noted above the amino acid residues of RTB critical for reassociation are remote from amino acid residues in subdomains 1α and 2γ (Rutener and Robertus, 1991). The stability of the recombinant heterodimers at high dilution samples as measured in both the modified ricin ELISA and cell cytotoxicity assay implies disulfide bond formation between RTA Cys259 and RTB Cys4 (Lewis and Youle, 1986).

Cell intoxication functions were not eliminated in the double-site mutants in contrast to the report of Newton et al. (1992). In their study, Xenopus expressed K40M/N46G/N255G was mixed with RTA and tested for uptake and cytotoxicity to mouse macrophages. No purification or quantification of heterodimer was done. We have used partially purified reagents and carefully measured heterodimer concentration. While potency was reduced 1.5–2 logs, the ID₅₀ was 3–4 logs lower than the background RTA cytotoxicity. Further, the correspondence of heterodimer potency with cell binding activity suggests even minimal galactose affinity of the molecule can provide adequate galactose binding-dependent function both on the cell surface and intracellularly in agreement with similar observations on chemically blocked ricin (Goldmacher et al., 1992; Grossbard et al., 1992).

The present study should provide a basis for further investigations on the possible identification of a third lectin site of ricin and provide a framework for initial construction of ricin-based fusion molecules (Frankel et al., 1995b).
Library Department, for molecular graphics analysis. This work was supported by NIH grant R01CA54116 to A.F. and ACS grant J781 to E.T.

References


Received August 30, 1995; revised January 15, 1996; accepted January 20, 1996