Expression of ricin B chain in *Spodoptera frugiperda*

Arthur FRANKEL,*† Hannah ROBERTS,* Lawrence AFRIN,* Joseph VESELY† and Mark WILLINGHAM†

Departments of *Medicine and †Pathology, Medical University of South Carolina, Charleston, SC, U.S.A.

DNA encoding ricin B chain was derived from preproricin genomic DNA and ligated into the baculovirus transfer vector, pAcGP67A. Co-transfection of *Spodoptera frugiperda* Sf9 cells with BaculoGold DNA was followed by limiting dilution purification of recombinant baculovirus. Infection of Sf9 cells at a multiplicity of infection of 1 in the presence of 25 mM lactose produced 3 mg/l of soluble, glycosylated, 34 kDa protein

immunoreactive with monoclonal and polyclonal antibodies to ricin B chain. The recombinant ricin B chain had similar lectin-binding activity to plant ricin B chain. The recombinant protein reassociated with ricin toxin A chain similarly to ricin toxin B chain and the recombinant heterodimers had similar cell cytotoxicity to ricin.

INTRODUCTION

Ricin, the toxic lectin isolated from the castor bean plant *Ricinus communis*, is a 65 kDa glycoprotein containing an A chain (RTA) and a B chain (RTB) linked by a disulphide bond [1]. Ricin is extremely toxic to eukaryotic cells. RTB is a galactose-specific lectin that mediates binding of the toxin to the surface of mammalian cells. After binding and internalization of ricin, RTA translocates across intracellular vesicle membranes to the cytosol, where it catalytically inactivates 60 S ribosomal subunits and thereby inhibits protein synthesis and causes cell death [2].

There are two lectin binding sites on RTB which have been reported to have similar affinities ($K_a = 10^{-4}$ M) [3] or very different affinities ($K_a = 10^{-3}$ M and 10^{-4} M) [4]. X-ray diffraction analysis of lactose-saturated ricin crystals suggested that the sugar binding sites are at the two ends of the molecule and involve amino acid residues D22, N46, Q35 and W37 in the $\alpha 1$ subdomain and D234, N255 and Y248 in the $\gamma 2$ subdomain of RTB [5]. Mutational analysis, using variant recombinant RTBs produced in monkey COS-M6 cells and *Xenopus* oocytes, also produced divergent results. Vitetta and Yen [6] reported that modification of asparagine 255 to alanine, ablated lectin activity, while Wales et al. [7] showed that modification of asparagine 255 and lysine 40 and asparagine 46 was required to block binding to galactose.

Immunotoxins, consisting of monoclonal antibodies reactive with neoplastic cells conjugated to RTA, have been produced and tested *in vitro*, in animal models *in vivo*, and in patients with chronic lymphocytic leukemia, ovarian carcinoma, breast carcinoma, leptomeningeal carcinomatosis, metastatic melanoma, colorectal carcinoma, cutaneous T-cell lymphoma and B-cell lymphomas [8]. Many of these RTA conjugates have been shown to be less potent than ricin conjugates [9]. RTB may enhance toxicity by facilitating internalization of the molecule to an intracellular compartment from which RTA may escape more easily to the cytosol [10]. Ricin immunotoxins can only be used *in vivo* if the nonspecific lectin function of the RTB is reduced. While this has been done chemically with some success [11], expression of recombinant variant RTB with genetically-blocked lectin function would permit construction of reproducible well-

defined blocked ricin immunotoxins. The role of residual galactose-binding in RTB enhancement of immunotoxin-cytotoxicity could also be determined. To date, the microgram quantities of variant RTBs have been insufficient to permit quantitative coupling to monoclonal antibodies.

RTB has been cloned and expressed in *Escherichia coli* [12], *Saccharomyces cerevisiae* [13], *Xenopus laevis* oocytes [14] and Cos-M6 cells [15]. Variant RTBs have been produced in *Xenopus* oocytes and COS-M6 cells. In each case only nanogram-microgram quantities of recombinant protein were generated. We chose to express recombinant RTB (rRTB) using recombinant baculovirus in *Spodoptera frugiperda* Sf9 insect cells, because the baculovirus system has been reported to produce milligram quantities of heterologous recombinant protein with proper folding and post-translational modifications including N-glycosylation [16].

MATERIALS AND METHODS

Materials

Restriction endonucleases and T4 ligase were obtained from Promega (Madison, WI, U.S.A.). 35 S-methionine, 32 P-dCTP, 35 S-dATP and 3 H-leucine were obtained from Amersham (Arlington Heights, IL, U.S.A.). Rabbit anti-ricin antibody, alkaline phosphatase conjugated goat anti-(rabbit IgG), alkaline phosphatase conjugated goat anti-(mouse IgG), asialofetuin, caesium chloride, α -lactose, glycerol, Bromophenol Blue, agarose, sodium phosphate, acrylamide, BSA, Bistris, EDTA, ammonium sulphate, tunicamycin, boric acid, SDS, NaCl, sodium azide, serum-free and protein-free hybridoma medium and *p*-nitrophenylphosphate were from Sigma (St. Louis, MO, U.S.A.). EX-CELL400 medium was obtained from JRH Scientific (Lexena, KS, U.S.A.). Sf9 insect cells, TMNFH medium, BaculoGold DNA and pAcGP67A transfer vector were from PharMingen (San Diego, CA, U.S.A.). Pansorbin was obtained from Calbiochem (San Diego, CA, U.S.A.). Prep-A-Gene DNA and plasmid purification matrices, Coomassie R-250, low molecular weight prestained protein standards, Whatman paper, Tween-20, β -mercaptoethanol and nitrocellulose paper were obtained from BioRad (Hercules, CA, U.S.A.). The Sequenase kit for

Abbreviations used: RTB, ricin toxin B chain; RTA, ricin toxin A chain; rRTB, recombinant ricin toxin B chain; moi, multiplicity of infection; β -ME, β -mercaptoethanol.

† To whom correspondence should be addressed at Hollings Cancer Centre Rm 306, MUSC, 171 Ashley Avenue, Charleston, SC 29425, U.S.A.

dideoxy sequencing was obtained from USB (Cleveland, OH, U.S.A.). The Random Primer labelling kit was obtained from Stratagene (La Jolla, CA, U.S.A.). X-ray film was from Kodak (Rochester, NY, U.S.A.). Dialysis membranes were from Spectrum Medical Industries (Los Angeles, CA, U.S.A.). Hybridomas producing P2 and P10 murine monoclonal antibodies to RTB and purified α BR12 murine monoclonal antibody to RTA were gifts of Dr. Walter Blattler, ImmunoGen (Cambridge, MA, U.S.A.). RPMI1640 medium, leucine-free RPMI1640, penicillin, streptomycin, Dulbecco's PBS, fetal bovine serum, and dialysed fetal bovine serum were obtained from GIBCO BRL (Grand Island, NY, U.S.A.). 3M Emphaze Biosupport medium ABl azlactone functionality bis-acrylamide was obtained from Pierce (Rockford, IL, U.S.A.). Triethylamine was obtained from Aldrich (Milwaukee, WI, U.S.A.). Biotinylated goat anti-(mouse Ig), biotinylated goat anti-(rabbit Ig), avidin-alkaline phosphatase Vectastain kit for Western blots, and alkaline phosphatase substrate kit for Western blots were obtained from Vector Laboratories (Burlingame, CA, U.S.A.). EIA plates, round-bottomed and flat-bottomed 96-well plates were from Costar (Cambridge, MA, U.S.A.). Liquid scintillation fluid was Ready-safe from Beckman (Fullerton, CA, U.S.A.). Plant RTB, ricin and RTA were obtained from Inland Laboratories (Austin, TX, U.S.A.).

Construction of pAcGP67A-RTB

pAKG plasmid encoding preproricin DNA [17] was a gift of Dr. Robert Weaver, University of Kansas. The plasmid DNA was used to transform INV α F' *E. coli* cells, and fresh plasmid DNA was prepared using the alkaline lysis method and purified on a silica-based matrix (Prep-A-Gene) following the manufacturer's instructions. Oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer and desalted with butan-1-ol. The 5'-oligonucleotide contained nine nonsense bases, a *Bam*HI site, an extra thymidine to place the RTB codons in the correct reading frame, and the coding sequence for the N-terminus of RTB. The 5'-oligonucleotide was lengthened to modify codon 6 GAT to GAC to remove an internal *Bam*HI site. The 3'-oligonucleotide contained nine nonsense bases, an *Eco*RI site, two stop codons and DNA encoding the C-terminal amino acid residues of RTB. The 3'-oligonucleotide was also lengthened to modify codon 239 GAT to GAC to remove another internal *Bam*HI site. The PCR product was purified on the Prep-A-Gene matrix, restricted with *Bam*HI and *Eco*RI, repurified on Prep-A-Gene matrix and subcloned in *Bam*HI-*Eco*RI restricted pAcGP67A. Sequence integrity was confirmed by double-stranded dideoxy-sequencing using the Sequenase kit and following the manufacturer's instructions. pAcGP67A-RTB DNA was then purified by caesium chloride density-gradient centrifugation.

Isolation of recombinant baculovirus

The Sf9 *S. frugiperda* ovarian cell line was maintained on TMNFH medium supplemented with 10% fetal calf serum. pAcGP67A-RTB DNA (2 μ g) was co-transfected with 0.5 μ g of BaculoGold AcNPV DNA into 2×10^6 Sf9 insect cells following the recommendations of the supplier. On day 5 post-transfection, the medium was centrifuged and the supernatant tested in a limiting dilution assay with Sf9 cells. 2×10^4 Sf9 cells were incubated with 10-fold dilutions of supernatant in 96-well plates. 8 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 blocked with Blotto and reacted with random primer 32 P-dCTP-labelled RTB DNA. After hybridization for 16 h at 67 °C, the dot-blot membrane was washed with $0.1 \times (150 \text{ mM NaCl}/15 \text{ mM sodium citrate})/1\%$ SDS, dried and exposed to X-ray film. Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to the 10^{-7} dilution were positive. Three rounds of selection were required. Recombinant virus in the supernatants was then amplified by infecting Sf9 cells at a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression of B chain in Sf9 cells

We determined the optimal moi and harvest-time for secretion of rRTB. 2×10^6 Sf9 cells plated into wells in 6-well plates in TMNFH were infected with recombinant baculovirus at moies of 0.01–10. Media was harvested at three days and assayed for rRTB by the asialofetuin e.l.i.s.a. (see below). Then 2×10^6 Sf9 cells were again seeded as above and infected at an moi of 1 and supernatants were harvested at 1, 2, 3, 4, 5 and 6 days post-infection and again assayed for rRTB. An aliquot of infected cells at day 3 post-infection was assayed for surface- and intracellular-rRTB by immunofluorescent staining as described below. 2×10^6 Sf9 cells in EX-CELL400 with or without 25 mM α -lactose were infected in spinner flasks at an moi of 1 and media and supernatants were collected at day 5.

Purification of rRTB

Media supernatants were adjusted to 0.01% sodium azide and maintained through all purification steps at 4 °C. The supernatants were concentrated 10-fold by vacuum dialysis, centrifuged at 3000 *g* for 10 min to remove precipitate, dialysed against 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA, 0.01% sodium azide/25 mM α -lactose (NTEAL), and ultracentrifuged at 100000 *g* for 1 h. The final supernatant was loaded onto a P2 monoclonal antibody-acrylamide column. Hybridoma cells producing P2 monoclonal anti-RTB antibody were grown in serum-free hybridoma medium. Antibody was isolated by precipitation with 50% ammonium sulphate, centrifugation at 12000 *g* for 1 h, resuspension in PBS, centrifugation at 3000 *g* for 10 min, dialysis against PBS, lyophilization and redialysis against PBS and filtration through 0.45 μ m low protein-binding Gelman Acrodisc filters. P2 (18 mg) was coupled to 3M Emphaze Biosupport Medium ABl azlactone functionality bis-acrylamide matrix following the supplier's instructions. The antibody (60% or 10 mg) was covalently coupled. The affinity-column was washed with NTEAL and rRTB eluted either with 0.1 M glycine, pH 2.8/3.5 M MgCl_2 /0.1 M triethylamine, pH 11.5/0.1 M triethylamine, pH 11.0, or 0.1 M triethylamine, pH 10.5. The acid eluant was immediately neutralized with 1 M Tris, pH 10, and the alkaline eluants were immediately neutralized with 1 M sodium phosphate, pH 6.8.

Cell pellets were suspended in 1/20 volume starting cell culture of 20 mM Tris, pH 7.9/0.5 M NaCl, 1 mM phenylmethanesulphonyl fluoride plus 2 μ g/ml aprotinin/1.5 μ g/ml leupeptin/1.5 μ g/ml pepstatin A/0.1% Triton X-100. The cells were frozen at -70 °C for 10 min, thawed and centrifuged at 260000 *g* for 60 min at 4 °C. The supernatant was assayed for rRTB by the asialofetuin e.l.i.s.a., and rRTB was purified from the extract by dialysis into NTEAL, ultracentrifugation at 100000 *g* for 60 min and application to P2-acrylamide as described above.

Molecular weight and charge of rRTB

Aliquots of rRTB-containing samples during various stages of purification, and prestained low molecular weight protein standards, were mixed with reducing or nonreducing 2×SDS sample buffer, placed in boiling water for 4 min, loaded on 15% SDS/PAGE and electrophoresed for 90 min. Gels were stained with 0.15% Coomassie Blue R-250 in 50% methanol/10% acetic acid, and destained initially with 50% methanol and 10% acetic acid and then with 7% acetic acid/5% methanol. Gels were dried under cellophane and scanned with the Apple One Scanner. Densitometric analysis was performed using Ofoto and Image 1.74 software.

Supernatant, after vacuum dialysis and centrifugation at 3000 *g* for 10 min, was dialysed into 25 mM Tris, pH 8/1 mM EDTA. Dialysed supernatant or plant RTB in 25 mM Tris, pH 8/1 mM EDTA was applied to an Econo-Pac Q cartridge anion-exchange matrix (BioRad) and eluted with a 0–500 mM NaCl gradient in 25 mM Tris, pH 8/1 mM EDTA. Fractions were assayed by the asialofetuin e.l.i.s.a.

Supernatant, after vacuum dialysis and centrifugation at 3000 *g* for 10 min, was dialysed into NTEAL and ultracentrifuged at 100000 *g* for 1 h. Half of the material was reduced for 30 min with 5% β -mercaptoethanol (β -ME) and applied to an 80 × 2.5 cm column of Bio-Gel P-100 medium-grade acrylamide matrix (BioRad) equilibrated in NTEAL plus 0.1% β -ME. The column was run at 0.4 ml/min and 5.9 ml fractions collected. The other half of the material was applied to an 80 × 2.5 cm column of Bio-Gel P-100 equilibrated in NTEAL and the column was run at 0.4 ml/min and 5.9 ml fractions collected. Control proteins applied to the columns included carbonic anhydrase, BSA and plant RTB, both reduced with 5% β -ME and non-reduced. Fractions were assayed for rRTB by the asialofetuin assay.

Glycosylation of rRTB

Two T-25 flasks were inoculated with 5 × 10⁶ Sf9 cells, 10 ml TMNFH and recombinant baculovirus at an moi of 1. After 48 h, the medium in one flask was changed to TMNFH plus 10 μ g/ml tunicamycin. After 60 h, the medium in both flasks was changed to Grace's medium without methionine, supplemented with 10% dialysed fetal bovine serum. Again, 10 μ g/ml tunicamycin was added to one flask. After 2 h, 250 μ Ci ³⁵S-methionine (1000 mCi/mmol) was added to each flask. Four hours later, the medium was removed and replaced with PBS. One of the flasks again contained 10 μ g/ml tunicamycin. After an additional 6 h of incubation, the cells and supernatants from each flask were harvested. Cells were suspended in 150 mM NaCl/1% NP40/0.5% DOC/0.1% SDS/50 mM Tris, pH 8/25 mM α -lactose (RIPAL). After freezing and thawing, cell extracts and supernatants were centrifuged at 22000 *g* for 10 min. Pansorbin fixed *Staphylococcus aureus* Cowan strain was prewashed with 3% BSA in PBS and then incubated with cell extracts and supernatants to remove non-specifically-binding labelled materials. The metabolically-labelled solutions were then reacted with Pansorbin pretreated with either rabbit anti-ricin antibody or non-specific rabbit serum. The Pansorbin was then pelleted and washed with RIPAL twice and RIPAL plus 500 mM NaCl once. The pellets were then boiled in reducing sample buffer and the samples were run along with prestained low molecular weight standards on a 15% SDS/PAGE. The gel was fixed briefly with 10% acetic acid, soaked for 1 h in Enlightening, dried and then exposed to X-ray film with enhancing screens for 1–3 days at –70 °C and developed.

N-terminal sequence of rRTB

Protein was lyophilized and adsorbed on an Immobilon poly(vinylidene difluoride) membrane and desalted and sequenced. Sequence analysis was done on a ABI477A protein sequencer with an in-line phenylthiohydantoin analyser ABI120A.

Immunological properties of rRTB

Infected Sf9 cells were washed with PBS and attached to polyllysine-coated tissue-culture dishes and centrifuged at 2000 *g* for 10 min. The cells were then either incubated live at 4 °C or fixed with 3.7% formaldehyde in PBS followed by 0.1% Triton X-100 in PBS at room temperature. Then the cells were washed with 2 mg/ml BSA in PBS and incubated in PBS plus BSA with 10 μ g/ml monoclonal antibody P10 reactive with RTB. The incubation was done at 4 °C for live cells and at room temperature for fixed cells. The cells were then washed with PBS and incubated with affinity purified goat anti-(mouse Ig) conjugated to rhodamine (ImmunoResearch) at 25 μ g/ml for 30 min either at 4 °C for live cells or at room temperature for fixed cells. The cells were then washed in PBS and post-fixed in 3.7% formaldehyde in PBS, mounted under a #1 coverslip in glycerol–PBS (90:10) and examined under a Zeiss Axioplan epifluorescence microscope.

Aliquots of rRTB, plant RTB and prestained low molecular weight standards were mixed with reducing or nonreducing 2×SDS sample buffer, boiled for 4 min, loaded on a 15% SDS/PAGE, and electrophoresed for 90 min. Gels, Whatman 3M #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 Trans-blot cell (BioRad). After electrophoresis at 15 V for 20 min, the nitrocellulose was blocked with 10% Carnation's nonfat dry milk/0.1% BSA/0.1% Tween-20/0.02% sodium azide. The blot was then washed with PBS plus 0.05% Tween-20 and PBS, incubated with P10 monoclonal anti-RTB antibody at 10 μ g/ml in PBS for 1 h, washed again, incubated with biotinylated goat anti-(mouse Ig) at 1:500 dilution in PBS for 1 h, washed again and developed with the avidin-alkaline phosphatase substrate kit, following the manufacturer's recommendations.

Monoclonal antibody P10 (100 μ l) at 5 μ g/ml in PBS was incubated in Costar EIA microtitre wells overnight at 4 °C. The wells were then washed with PBS plus 0.1% Tween 20, blocked with 3% BSA in PBS/Tween, rewashed, incubated with dilutions of plant RTB- or rRTB-containing samples, rewashed, incubated with rabbit anti-ricin antibody 1:400 in PBS, washed again, incubated with alkaline phosphatase conjugated goat anti-(rabbit Ig) at 1:1000 in PBS, 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.

Lectin activity of rRTB

A 100 μ l volume of 1 μ g/ml asialofetuin in PBS was added to wells of a Costar EIA plate and incubated overnight at 4 °C. The wells were then washed with PBS/0.1% Tween-20, blocked with 3% BSA/PBS/0.01% sodium azide and rewashed. Dilutions of plant RTB or rRTB in 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA 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/0.01% 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; finally, the wells were washed and reacted with 1 mg/ml nitrophenylphosphate (Sigma) in 50 mM

diethanolamine buffer, pH 9.6, and measured in a microtitre plate reader at 405 nm.

Samples of rRTB or plant RTB in 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA, 0.01 % sodium azide (NTEA) were loaded on a lactosyl acrylamide matrix (Pierce), washed with NTEA, and lactose binding protein eluted with NTEA plus 50 mM lactose. Fractions were assayed for absorbance at 280 nm and asialofetuin binding as described above.

Reassociation with plant RTA to form heterodimers

A 30 µg portion of rRTB or plant RTB was mixed with 90 µg of plant RTA in NTEA plus 50 mM lactose shaking overnight at room temperature. The reaction mixture was then analysed by a ricin e.l.i.s.a. Wells of an EIA plate were coated with 1 µg/ml asialofetuin 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/Tween, re-washed and incubated with dilutions of ricin or re-associated heterodimers. The wells were again washed, incubated with αBR12 monoclonal antibody at 1 µg/ml in PBS, re-washed and incubated with alkaline phosphatase-conjugated goat anti-(mouse Ig) at 1:500 in PBS, 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 microtitre plate reader. Reassociated mixtures were also analysed by non-reducing SDS/PAGE followed by immunoblots with αBR12 anti-RTA monoclonal antibody and P10 anti-RTB monoclonal antibody. Densitometric scanning using the Apple One Scanner was done to quantify shift of immunoreactive material from 30 kDa to 60 kDa.

Cytotoxicity of recombinant heterodimers

1.5×10^6 HPB-MLT human T leukaemia cells (a gift from Dr. Daniel Vallera, University of Minnesota) in 100 µl were placed in 96-well flat-bottomed plates in leucine-poor RPMI1640 containing 10 % dialysed fetal bovine serum. 50 µl of ricin, plant RTA-RTB (recombinant heterodimers) and plant RTA, plant RTB and rRTB alone at varying concentrations were added in the same medium and the cells incubated at 37 °C in 5 % CO₂ for 24 h. ³H-leucine, 1 µCi per well (120 mCi/mmol), in 50 µl of the same medium was added and incubated for 4 h. Cells were then harvested with a Skatron Semiautomatic Cell Harvester onto glass-fibre filter mats. The filters were dried, mixed with 3 ml of liquid scintillation fluid and counted in an LKB-Wallac liquid scintillation counter gated for ³H. 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 α-lactose. The ID₅₀ was the concentration of protein which inhibited protein synthesis by 50 % compared with control.

RESULTS AND DISCUSSION

Expression and secretion of rRTB

The transfection supernatant contained recombinant baculovirus at a titre of 5×10^2 /ml. The supernatant from wells on the first limiting dilution assay had a titre of 10^7 /ml. Subsequent supernatant continued to give titres of approximately 10^7 /ml. After amplification of the virus, the 100 ml supernatants contained at least 10^7 infectious particles/ml. Thus, only one or two rounds of selection are necessary with the BaculoGold transfection system.

These results are similar to previously published observations [18]. Infectious virus produced in the transfection must contain recombinant sequences to complement the lethal deletion in the ORF1629 of the BaculoGold AcNPV DNA.

Sf9 cells were infected with varying mois (0.1, 0.5, 1 and 2) of recombinant baculovirus and supernatants were harvested at three days. The levels of rRTB were tested by asialofetuin e.l.i.s.a. and antibody e.l.i.s.a. and gave comparable results. The secreted rRTB levels were 10 ng/ml for 0.1 moi, 20 ng/ml for 0.5 moi and 50 ng/ml for mois of 1 and 2. The e.l.i.s.a. assays were sensitive to 5 ng/ml plant RTB. These observations confirm those of others that mois of greater than 1 are needed for maximal protein production [19].

Sf9 cells were then infected with an moi of 1 with recombinant baculovirus, and supernatants harvested at 1, 2, 3, 4 or 5 days. Secreted protein rose from 50 ng/ml at 24 h to 100 ng/ml at 48 h, 200 ng/ml at 72 h and 400 ng/ml at 96 and 120 h. The optimal time of harvest was therefore day 4–5. This observation suggests that the secreted protein is stable and not proteolytically degraded. Other investigators have reported maximal protein levels at day 2–3 and have observed proteolysis which can be partially abated with pCMBS [20] or PMSF (P. Arnaud, personal communication).

Infection of Sf9 insect cells and *Trichoplusia ni* insect cells with baculovirus at an moi of 1 and harvesting at 4 days. Levels were 300 ng/ml for both cell types. We were unable to confirm higher levels of expression in *T. ni* cells as previously reported [21].

Because our observed secretion of rRTB of 300 ng/ml was 1–2 orders of magnitude lower than reports of secretion of recombinant proteins with baculovirus infected Sf9 cells, we examined conditions that might enhance the stability or secretion of rRTB. We chose initially to add 25 mM α-lactose to the medium. Lactose is known to stabilize both plant RTB [22] and renatured *E. coli*-expressed rRTB [12]. The presence of α-lactose in the medium increased levels of rRTB from 300 ng/ml to 3000 ng/ml. Either the sugar stabilizes extracellular rRTB or enhances secretion.

Intracellular levels of rRTB were measured by extracting cell pellets in 0.5 M NaCl/0.1 % Triton X-100/20 mM Tris, pH 7.9, with proteinase inhibitors. Both in the presence and absence of α-lactose, intracellular levels of rRTB were 1 µg/10⁶ cells. These levels are 3-fold lower than secreted levels of rRTB in the presence of lactose and suggest the GP67A leader-ADP-RTB is being secreted efficiently under these conditions.

Measurement of surface and intracellular rRTB by immunofluorescent staining shows high levels of intracellular rRTB and no surface rRTB (Figure 1). These results suggest the GP67A leader-ADP-RTB is processed efficiently.

Purification of rRTB

After concentration, dialysis and ultracentrifugation of infected Sf9 supernatants, measurements of rRTB and total protein were made. Based on absorbance at 280 nm and density on Coomassie-stained gels, each ml of culture supernatant contained 6 mg of protein. Thus, rRTB represents about 0.05 % of the total protein. The medium itself is not responsible for the additional protein as EX-CELL400 alone treated identically showed no bands on Coomassie-stained gels. Supernatants of Sf9 cells infected with either recombinant baculovirus or 'wild-type' AcNPV had 6 mg/ml of protein, suggesting the day 5 supernatants are composed primarily of lysed insect cell proteins.

Size-exclusion chromatography and anion-exchange chromatography led to partial purifications of 10-fold and 5-fold, respectively. Size-exclusion chromatography was performed with

Figure 1 Immunofluorescence of Sf9 insect cells infected with recombinant baculovirus

Cells were attached to poly-lysine coated tissue culture dishes, and either directly incubated with antibody as described below or first fixed with 3.7% formaldehyde in PBS followed by 0.1% Triton X-100 in PBS, washed with 2 mg/ml BSA in PBS and then PBS plus BSA plus monoclonal antibody P10 at 10 μ g/ml. The cells were washed with PBS and incubated with affinity-purified goat anti-(mouse Ig) coupled to rhodamine (Jackson ImmunoResearch) at 25 μ g/ml, rewashed and postfixed in 3.7% formaldehyde in PBS. (a) Cells examined at 200 $\times$ under phase contrast. (b) Cells examined for fluorescence prefixed with formaldehyde and treated with Triton X-100. (c) Cells examined for fluorescence, stained live and fixed. Control non-infected cells showed no fluorescence (not shown). Live cells stained prior to formaldehyde fixation showed no fluorescence. Cells fixed with formaldehyde followed by Triton X-100 and stained showed cytosolic fluorescence.

an acrylamide matrix to avoid interactions between the rRTB lectin and a galactose-based Sepharose matrix. When the infected Sf9 concentrated, dialysed and ultracentrifuged supernatants were reduced with β -ME and applied to a Bio-Gel P-100 column equilibrated with buffer containing 0.1% β -ME, the rRTB eluted at about 30 kDa, whereas the majority of insect cell proteins eluted in a broad peak around 60 kDa.

Reduction of the insect supernatants was found to be necessary to eliminate rRTB homodimers. Anion-exchange chromato-

graphy was performed on concentrated supernatants dialysed into buffer lacking NaCl. The rRTB eluted from the Q-acrylamide matrix at 250 mM NaCl in Tris pH 8. Unfortunately, rRTB was not stable in low-salt buffers and two-thirds of the starting rRTB was lost by ultracentrifugation prior to loading the column. rRTB and plant RTB bound poorly to the Q matrix at NaCl concentrations of 10–25 mM at pH 8. Combining size-exclusion chromatography and anion-exchange chromatography resulted in purifications of 50-fold, but the rRTB still represented only 2.5% of the total protein.

Lactosyl acrylamide affinity-chromatography of infected Sf9 supernatants after concentration and dialysis into non-lactose containing solution led to 80–90% purification of rRTB. About half of the applied plant RTB or rRTB bound to the matrix and was eluted with 50 mM lactose. The eluted rRTB yielded a single band on Coomassie-stained gels and immunoblots with monoclonal antibody to RTB (Figure 2) and had asialofetuin-binding indistinguishable from plant RTB. We sought a non-lectin binding method of purification, so that purification of non-sugar binding variants of rRTB could be prepared and purified for analysis.

Monoclonal antibody P2, reactive with RTB, was coupled to acrylamide and reacted with plant RTB. Over 90% of the plant RTB bound in the presence of 50 mM NaCl/25 mM Tris, pH 8, or in the presence of EX-CELL400. No significant elution of plant RTB was found using acid or high-salt conditions. However, in the presence of alkaline conditions (pH 11 or greater) plant RTB was quantitatively recovered. The material was immediately neutralized and tested for lectin activity. Full bioactivity was found. Subsequently, we tested rRTB-containing supernatants and found > 90% binding of rRTB to the column and full elution with triethylamine, pH 11. The final eluant was 60–80% pure, based on Coomassie-stained gels (Figure 3). The major contaminant was the P2 monoclonal antibody which leached to a small degree from the matrix during alkaline treatment. The rRTB had 81–100% of the asialofetuin-binding of plant RTB. Other investigators have documented that some proteins elute better under alkaline than under acid conditions [23].

Chemical characterization

The molecular weight of insect rRTB is estimated as 33 kDa based on Coomassie-stained gels and size-exclusion chromatography. The pI is estimated to be about 4.8 based on its similar pattern of salt elution from the Q anion-exchange matrix as plant RTB. Plant RTB has been reported to have a pI of 4.8 [24]. Glycosylation was documented by immunoprecipitation of rRTB produced in the presence and absence of tunicamycin (Figure 4). In the presence of 10 μ g/ml of tunicamycin, rRTB had a molecular weight of 29 000 kDa. In the absence of tunicamycin, metabolically labelled rRTB had a molecular weight of 33 kDa. rRTB synthesized and processed by insect cells should have a molecular weight of 29.4 kDa. Thus, approximately 4 kDa of sugars are attached. The normal *N*-linked oligosaccharides of insect proteins have the composition $\text{Man}_6\text{GlcNac}_2$ or $\text{Man}_3\text{GlcNac}_2$ [25]. There are two *N*-glycosylation sites on RTB. $\text{Man}_6\text{GlcNac}_2$ has a molecular weight of 2.062 kDa and $\text{Man}_3\text{GlcNac}_2$ has a molecular weight of 0.982 kDa. We suggest that insect rRTB has two high mannose oligosaccharides of the $\text{Man}_6\text{GlcNac}_2$ type.

The *N*-terminal sequence of insect rRTB was ADPADVCM. The predicted cleavage-site at the C-terminal end of the GP67A leader sequence is between the adjacent alanines in HSAFAADP. Thus, the insect rRTB has complete and proper processing of the

Figure 2 Lactosyl-acrylamide affinity-chromatography of infected Sf9 supernatants

15% reducing SDS/PAGE of recombinant insect RTB purified from dialysed concentrate day 5 post-infection at $\text{moi} = 1$ Sf9 culture supernatants bound to lactosyl acrylamide in 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA/0.01% sodium azide and eluted in the same buffer plus 50 mM α -lactose. (a) Coomassie stained gel: lane 1, low molecular weight prestained BioRad protein standards; lane 2, rRTB. (b) Immunostaining with monoclonal antibody P10 of nitrocellulose transferred gel. Lane 1, low molecular weight prestained BioRad protein standards; lane 2, rRTB.

Figure 3 Monoclonal antibody P2 coupled to acrylamide and reacted with plant RTB

15% reducing SDS/PAGE of recombinant insect RTB purified from dialysed concentrate day 5 post-infection at $\text{moi} = 1$ Sf9 culture supernatants bound to P2 monoclonal antibody-acrylamide matrix in 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA/0.01% sodium azide/50 mM α -lactose and eluted with 0.1 M triethylamine, pH 11, and neutralized immediately with 1 M sodium phosphate buffer, pH 6.8. (a) Coomassie stained gel: lane 1, low molecular weight BioRad protein standards; lane 2, rRTB. (b) Immunostaining with monoclonal antibody P10 of nitrocellulose transferred gel: lane 2, low molecular weight prestained BioRad protein standards; lane 2, rRTB.

leader, leaving a tripeptide ADP at the N-terminus of the secreted rRTB. ADVCM are the first five amino acid residues of plant RTB.

The insect rRTB was stable at 4 °C and -20 °C for 3 weeks and was not lost by centrifugation at 100 000 *g* for 1 h or passage through Gelman 0.45 μm low-protein-binding filters. There was no increased instability by storage in buffers without lactose. However, buffers with low ionic strength (< 50 mM NaCl) led to protein denaturation within 48–72 h.

Lectin activity

rRTB bound asialofetuin and lactose on a molar basis 80–100% as well as plant RTB. These results are based on properties of

Figure 4 Immunoprecipitation of rRTB in the presence and absence of tunicamycin

Autoradiogram of 15% reducing SDS/PAGE of ^{35}S -methionine-labelled cell extracts immunoprecipitated with rabbit anti-ricin (Sigma) and Pansorbin (Calbiochem). Arrows, low molecular weight BioRad protein standards: lane 1, tunicamycin (10 $\mu\text{g}/\text{ml}$) treated recombinant baculovirus-infected Sf9 cell extract immunoprecipitate; lane 2, non-tunicamycin-treated infected Sf9 cell extract immunoprecipitate.

Figure 5 Reassociation of rRTB with plant RTA

Immunoblot using monoclonal antibody P10 anti-RTB of 15% nonreducing SDS/PAGE of plant RTA, reassociated rRTB-plant RTA, and rRTB. Lane 1, 1 μg rRTB; lane 2, 1 μg reassociated rRTB-plant RTA; lane 3, 1 μg plant RTA; arrows in lane 4 indicate low molecular weight BioRad protein standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa). RTB immunoreactive material appears at 60 kDa after reassociation with plant RTA.

purified recombinant protein. The ability to produce milligram quantities of rRTB will permit careful assessment of sugar-binding for 'wild-type' and variant rRTBs containing modifications of specific amino acid residues. Adequate quantities of recombinant RTB proteins are now available for comparative studies in different laboratories, and potentially for efforts to crystallize rRTB and determine structure-function relationships.

Reassociation with plant RTA

Incubation of rRTB at 10^{-6} M with plant RTA at 3×10^{-6} M overnight at room temperature yielded 50% of the rRTB reassociated with RTA (Figure 5). Similar levels of reassociation were seen using plant RTB with plant RTA at the same concentrations. The heterodimer concentrations were quantitated by ricin e.l.i.s.a. The interactions critical for reassociation are present in insect rRTB including C-7, A-4, D-5, D-97, V-144, K-222, N-223, F-265, P-263, F-143 and F-221. The first three amino acid residues of RTB in ricin crystals have disordered structure and are floating in the solvent. The tripeptide ADP added in the insect rRTB presumably is also remote from the RTA-RTB interface. The recombinant heterodimer is stable, with presumably disulphide linkage between plant RTA C-259 and insect

Figure 6 HPB-MLT cell cytotoxicity

2×10^5 cells/well were incubated with varying concentrations of heterodimers or ricin subunits for 20 h at 36 °C, 5% CO₂ and 1 μ Ci/well of ³H-leucine added for 4 h and then cell lysates harvested on glass-fibre filters and washed. Filters were dried and counted in a liquid scintillation counter. (a) Proteins incubated with cells: ○, ricin; △, rRTB-RTA; □, plant RTB-RTA; ●, ricin plus 50 mM lactose; ▲, rRTB-RTA plus 50 mM lactose; ■, plant RTB-RTA plus 50 mM lactose. (b) Proteins incubated with cells: ●, ricin; △, rRTB; ○, rRTB-RTA; □, plant RTB-RTA; ▽, plant RTB; ×, plant RTA

rRTB C-7, because the material can be diluted over a 1000-fold without dissociation.

Cytotoxicity of recombinant heterodimers

The ID₅₀s of ricin, reassociated plant RTB-RTA, and reassociated rRTB-RTA were 1.2×10^{-12} M, 6×10^{-13} M and 9×10^{-13} M, respectively, in one experiment and 7×10^{-13} M for all three on a second experiment (Figure 6). The presence of 50 mM α -lactose increased the ID₅₀ 143-fold, 243-fold and 129-fold, respectively. Plant RTA, plant RTB and rRTB had ID₅₀s $> 10^{-9}$ M. Thus, the recombinant heterodimer was equally toxic as ricin and required sugar-binding to effect cytotoxicity. The recombinant RTB alone was completely non-toxic. The cytotoxicity of insect rRTB-RTA was similar to that described for *Xenopus* and COS-M6 rRTB-RTA heterodimers [14,15] and was 10-fold more cytotoxic than bacterial rRTB-RTA heterodimers [12]. Either RTB must be glycosylated for optimal heterodimer intoxication or the renatured bacterial rRTB was mis-folded in a way that inhibited toxicity. Since chemical modification of RTB oligosaccharides impairs heterodimer cytotoxicity [26,27], we suggest that glycosylation of RTB is needed for fully efficient toxin killing.

The preparation of milligram quantities of fully active

recombinant heterodimer makes possible genetic engineering of ricin similar to that reported previously for diphtheria toxin [28] and *Pseudomonas* exotoxin [29]. Fusion of new ligands to rRTB followed by reassociation with plant or recombinant RTA should provide a smaller more homogeneous product for therapeutic studies *in vitro* and *in vivo*.

CONCLUSION

We subcloned DNA encoding RTB in the baculovirus transfer vector, pAcGP67A [30], and co-transfected Sf9 cells with caesium chloride gradient-purified pAcGP67A-RTB DNA and BaculoGold DNA. Recombinant baculovirus was isolated by limiting-dilution analysis. Sf9 cells were then infected at an moi of 1 and supernatants and cells harvested at 120 h. This report documents that rRTB was expressed and secreted into the medium at 3 mg/l in Ex-Cell 400 serum-free medium supplemented with 25 mM lactose. rRTB was purified by vacuum concentration followed by dialysis using Spectrum 12000–14000 kDa cut-off cellulose membranes against 50 mM NaCl/25 mM Tris, pH 8/1 mM EDTA/0.01% sodium azide with ultracentrifugation at 100000 *g* for 1 h at 4 °C and affinity chromatography on a P2 anti-RTB monoclonal antibody–acrylamide column (UltraLink, Pierce) using 0.1 M triethylamine, pH 11.5, elution. The eluted rRTB was immediately neutralized with 1 M sodium phosphate, pH 6.8. Purity of 60–80% was obtained. Purified rRTB bound lactose and asialofetuin similarly to plant RTB. Furthermore, the rRTB re-associated with plant RTA equally as well as plant RTB and formed fully cytotoxic heterodimers. N-terminal sequencing of the rRTB confirmed proper processing by the insect cells of the gp67A leader peptide and the tripeptide (ADP) addition to the N-terminus of RTB due to linker DNA.

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