Ricin Toxin Contains at Least Three Galactose-Binding Sites Located in B Chain Subdomains 1α, 1β, and 2γ†

Arthur E. Frankel,*,‡ Chris Burbage,‡ Tao Fu,‡ Edward Tagge,§ John Chandler,§ and Mark C. Willingham||

Departments of Medicine, Surgery, and Pathology, Medical University of South Carolina, Charleston, South Carolina 29425

Received April 3, 1996; Revised Manuscript Received August 28, 1996⊗

ABSTRACT: Ricin toxin, the heterodimeric 65 kDa glycoprotein synthesized in castor bean seeds, consists of a cell binding lectin subunit (RTB) disulfide linked to an rRNA *N*-glycosidase protein synthesis inactivating subunit (RTA). While X-ray crystallography and equilibrium dialysis suggested two sugar-combining sites located in subdomains 1α and 2γ, biochemical and mutational analyses suggested the existence of a third lectin site. We performed oligonucleotide-directed mutagenesis on RTB cDNA to create mutants with modifications in subdomains 1α, 2γ, and either 1β or 2α. The triple-site mutant RTBs were expressed in insect cells. Partially purified recombinant proteins obtained from infected cell extracts and cell supernatants were characterized for asialofetuin and cell binding, immunoreactivites, ability to reassociate with RTA, and recombinant heterodimer cell cytotoxicity. Yields of both triple-site mutants were similar to the parent double-site mutant. Both mutants showed immunoreactivity with a panel of anti-RTB monoclonal and polyclonal antibodies. The triple-site mutant with modification of amino acid residues in subdomains 1α, 2α, and 2γ bound asialofetuin and cells similarly to the parent 1α, 2γ, subdomain mutant. In contrast, the 1α, 1β, 2γ subdomain triple-site mutant had a one and one-half log decrease in asialofetuin and cell binding relative to the parent double-site mutant. The 1α, 2α, 2γ triple-site mutant and 1α, 2γ parent protein had sugar binding which was inhibited by 3−27-fold by lactose and asialofetuin. Both triple-site mutants reassociated well with RTA. The 1α, 2α, 2γ triple-site mutant−RTA was equally cytotoxic to mammalian cells as the double-site mutant−RTA heterodimer. In contrast, the 1α, 1β, 2γ triple-site mutant−RTA was 25 times less toxic than the double mutant and 20 times more toxic than RTA alone. These data support a model for at least three lectin-binding subdomains in RTB.

The evolutionary selection of protein structure would be expected to favor conformations that match potential function. In the case of lectins, high avidity binding has often been derived from the interaction of multiple sugar-binding sites spaced at distances that would be present on potential target glycoconjugates. For example, the hepatic asialoglycoprotein receptor, a cell surface lectin, has three closely spaced monomeric sugar binding sites corresponding to oligosaccharides present on small circulating plasma glycoproteins. On the other hand, lectins that react with cell surfaces, such as influenza virus hemagglutinin, pertussis toxin, and mammalian mannose-binding protein, have multiple sugar-binding sites spaced further apart, spacing that would be unlikely to react with single glycoprotein molecules but would be expected on the bumpy, irregular surface of intact mammalian cells. In this study, we investigated whether the plant-derived lectin, ricin, also contains widely spaced binding sites indicative of evolutionarily selected cell surface binding domains.

Ricin toxin, isolated from the seeds of *Ricinus communis* plants, is one of the most poisonous substances known to man. The 65 kDa heterodimeric glycoprotein binds to cell surface galactose-terminated oligosaccharides via lectin-binding sites in RTB[1] (Baenziger & Fiete, 1979) and undergoes receptor-mediated endocytosis (Sandvig & Olsnes, 1982). After trafficking to the Golgi (van Deurs et al., 1987), the toxin is transported to a distal compartment (Wales et al., 1993) from which the intersubunit disulfide bond is reduced (Lewis & Youle, 1986). RTA then translocates to the cytosol and catalytically inactivates protein synthesis by hydrolysis of a specific adenine base from the 26S ribosomal RNA (Endo & Tsurugi, 1987). Galactose binding is important for cell binding and may be needed for internalization and intracellular trafficking of ricin. We initiated studies on the molecular nature of the lectin-binding sites of ricin both to further our knowledge of plant lectin structure−function relationships and to provide information for the design of ricin-based fusion toxins.

Previous competition experiments with synthetic methyl β-lactoside analogs and equilibrium dialysis measurements with [^{3}H]galactose and lactose demonstrated β-galactoside specificity of binding with low affinity constants of 10^3−10^4 M^{-1} for simple sugars (Rivera-Sagredo et al., 1991; Zentz et al., 1978; Houston & Dooley, 1982). In contrast, Scatchard plots of iodinated ricin binding to cells showed association constants of 10^7−10^8 M^{-1} (Sandvig et al., 1976). Thus, the data supported multiple low affinity sugar-binding sites on ricin interacting with complex oligosaccharides and cells to yield high affinity binding. Thus, we determined

† This research was supported by ACS Grant 98-80 (E.T.) and a grant from the Medical University of South Carolina (A.E.F.).

* Author to whom correspondence should be addressed: Hollings Cancer Center, Rm 311, 86 Jonathan Lucas St., Medical University of South Carolina, Charleston, SC 29425. Telephone: 803-792-1450. Fax: 803-792-3200. E-mail: frankeae@musc.edu.

‡ Department of Medicine.

§ Department of Surgery.

|| Department of Pathology.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1996.

[1] Abbreviations: RTB, ricin toxin B chain; RTA, ricin toxin A chain; rRNA, ribosomal RNA; moi, multiplicity of infection; RGD, arginine−glycine−aspartic acid sequence; CD, circular dichroism.

S0006-2960(96)00798-2 CCC: $12.00

that our experiments should define both the number and location of sugar-combining sites on ricin.

The X-ray crystallographic structure of ricin revealed two domains each with three subdomains each with similar folding and primary amino acid sequence which might bind sugars (Ruteber & Robertus, 1991). While all six subdomains had α-carbon chains forming a loop, twist, and hook, only four of the subdomains (1α, 1β, 2α, and 2γ) contained tripeptide kinks. Cocrystallization of 5 mM α-lactose with ricin showed sugar binding in the tripeptide kinks of subdomains 1α and 2γ. Thus, we reasoned that two to four subdomains offered similar structural regions for reaction with galactose.

To explore the participation of subdomain amino acid residues in sugar binding, we isolated cDNA encoding RTB and employed a baculovirus expression system to recover recombinant proteins for analysis of sugar binding (Frankel et al., 1996a). Vyas and colleagues (Vyas, 1991) had reported that group II carbohydrate-binding proteins including plant lectins, lysozyme, xylose/glucose isomerase, and others had shallow binding grooves featuring stacking interactions with aromatic residues and bidentate hydrogen bonds with aspartic acid, glutamic acid, asparagine, or arginine. Further, the reported X-ray crystal structure of ricin with lactose demonstrated proximity of Trp-37 and Tyr-248 to the B-face of the galactose moiety and alignment of Asp-22 and Asp-234 with the C3,C4 hydroxyls of galactose (Ruteber & Robertus, 1991). On the basis of the above, we chose to modify either aromatic ring residues or polar/charged residues near the kink in subdomains 1α and 2γ. Sixteen mutants were prepared and studied (Frankel et al., 1996a,b; unpublished experiments). Surprisingly, single-site mutants retained asialofetuin and cell binding within one log of wild type (Frankel et al., 1996a). We anticipated that if RTB had only two low affinity sugar-binding sites, the removal of one of these sites should drastically alter the mutant RTB affinity for complex oligosaccharides. Since this was not observed, we postulated three or more lectin sites. The double-site mutants had, in most cases, two log decreases in asialofetuin and cell binding but retained significant sugar binding that could be competed with lactose and asialofetuin (Frankel et al., 1996b; unpublished experiments). These results provided further evidence for a third lectin site. To confirm the presence of a third lectin site, we have prepared triple-site mutants. We now report the biological properties of two of these mutants—one with modifications in subdomains 1α, 2α, and 2γ and the other with modifications of residues in subdomains 1α, 1β, and 2γ. Only the triple-site mutant containing a change in an amino acid residue in 1β led to further loss of sugar binding. Our finding supports the hypothesis of at least three independent sugar-combining sites on ricin. Further, the lectin sites are distant from each othr unlike the monomer binding hepatic Gal/GalNac receptor (Lee, 1992). Instead, ricin resembles the cell surface binding influenza virus hemagglutinin, cholera toxin, pertussis toxin, and mammalian mannose-binding protein (Drickamer, 1995). The trivalent binding motif resembling the milkmaid's stool may provide optimal binding to an uneven oligosaccharide-rich cell surface. On the basis of these results, ricin fusion toxins must have genetic modifications of residues, at the minimum, in RTB subdomains 1α, 1β, and 2γ to reduce normal tissue toxicities.

MATERIALS AND METHODS

Selection of RTB Modifications. Connolly water-accessible surfaces of RTB subdomains 1β and 2α were plotted using Brookhaven coordinates from Rutenber and Robertus (1991) and SYBYL software on a Silicon Graphics Iris Indigo workstation. Aromatic ring residues projecting into the α-carbon kink in each subdomain were identified. Substitutions producing similar side-chain size and charge were examined.

Construction of Transfer Vectors Encoding Mutant RTBs. Site-specific mutagenesis was performed on single-stranded pUC119-RTB[W37S/Y248H] DNA using the Sculptor in vitro mutagenesis kit as previously described (Frankel et al., 1996a). Modifications were made at either the 1β or 2α subdomain to alter aromatic ring residues which provide van der Waals interactions between the protein and sugar. The *Bam*HI−*Eco*RI mutant RTB encoding DNA fragment was then subcloned into pAcGP67A plasmid (PharMingen, San Diego, CA) and used to transform INVαF′ *Escherichia coli* cells. Transfer vectors with mutant RTBs were then purified by cesium chloride gradient centrifugation.

Isolation of Recombinant Baculoviruses. pAcGP67A-mutant RTB DNAs (4 μg) were cotransfected with 0.5 μg of BaculoGold AcNPV DNA (PharMingen) into 2×10^6 Sf9 *Spodoptera frugiperda* insect cells as recommended by the supplier. On day 7 posttransfection, media were centrifuged and supernatants tested in limiting dilution assays with Sf9 cells as previously described (Frankel et al., 1996a). Positive wells were identified and supernatants reassayed by limiting dilution until all wells up to 10^{-8} 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 a multiplicity of infection (moi) of 0.1, followed by collection of day 7 supernatants.

Expression and Purification of Mutant RTBs. Recombinant baculoviruses were used to infect Sf9 cells at an moi of 5 in EX-CELL400 media (JRH Scientific, Lexena, KS) with 25 mM α-lactose in spinner flasks. Media supernatants and cell pellets containing mutant RTBs were collected day 6 postinfection. The supernatants were adjusted to 0.01% sodium azide, centrifuged at 3000*g* for 10 min, concentrated 15-fold by vacuum dialysis, recentrifuged at 3000*g* for 10 min, dialyzed into 50 mM NaCl, 25 mM Tris, pH 8, 1 mM EDTA, 0.01% sodium azide, and 25 mM α-lactose (NTEAL) at 4 °C, ultracentrifuged at 100000*g* for 1 h at 4 °C, and loaded onto a P2 monoclonal antibody−acrylamide column. The column was washed 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 were eluted with 0.1 M triethylamine hydrochloride, pH 11, and immediately neutralized with 1 M sodium phosphate, pH 4.8, and stored at −20 °C until assayed. Cell pellets were dissolved in 20 mM Tris-HCl, pH 8, 50 mM NaCl, 1% NP40, 1 mM PMSF, 2 μg/mL aprotinin, 1.5 μg/ml pepstatin, and 1.5 μg/mL leupeptin, frozen at −70 °C, thawed, centrifuged at 22000*g* for 15 min at 4 °C, dialyzed into NTEAL at 4 °C, and treated identically to dialyzed concentrated cell supernatants.

Chemical and Immunological Characterization of Mutant RTBs. Optical densities at 280 nm were determined. Aliquots were run on a reducing 15% SDS−PAGE, stained with Coomassie Blue R-250, and scanned on an IBAS

automatic image analysis system. Aliquots were also run on a reducing 15% SDS−PAGE and transferred to nitrocellulose using a Semi-dry Trans-blot cell (Bio-Rad, Hercules, CA), and immunoblots were performed with rabbit anti-ricin antibody as previously described (Frankel et al., 1996a,b). Antibody ELISAs using monoclonal anti-RTB antibodies P2, P8, or P10 as well coats were done on each mutant following the method previously reported (Frankel et al., 1996a,b). CD spectra were obtained by applying protein (8−100 μg/mL) in 0.1 M triethylamine/sodium phosphate, pH 8, in 1 mL total volume to a Aviv-60DS circular dichroism spectrometer, and typically five scans from 180 to 350 nm were summed. Trypsin sensitivity was assessed by mixing aliquots of trypsin (Sigma) with plant RTB or mutants (0.5 μg) at ratios of 1:100 to 1:1 in 50 μL of 0.1 M triethylamine/sodium phosphate, pH 8, and incubating for 30 min at 37 °C. The reactions were stopped with addition of sample buffer. Samples were boiled for 5 min and loaded on a reducing 15% SDS−PAGE. Peptides were transferred to nitrocellulose and immunoblotted with anti-RTB antibody as described above.

Lectin Activity of Mutant RTBs. Asialofetuin ELISAs and binding to KB cells in the presence or absence of 100 mM α-lactose or 100 μg/mL asialofetuin were performed as previously described (Frankel et al., 1996a). Briefly, KB human epidermoid carcinoma 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 washed with 2 mg/mL BSA in PBS and incubated in PBS plus BSA with 1 μg/mL plant RTB, RTB[W37S/Y248H], RTB[W37S/Y248H/Y78H], or RTB[W37S/Y248H/W160S] with or without 100 μg/mL asialofetuin for 30 min at 4 °C. The cells were again washed with PBS and incubated with rabbit anti-ricin antibody (Sigma; 1:100 in PBS plus BSA) for 30 min at 4 °C. The cells were rewashed with PBS and reacted with goat anti-rabbit Ig conjugated to rhodamine (Jackson Immunoresearch, West Grove, PA) at 25 μg/mL for 30 min at 4 °C. After a final wash with PBS, the cells were fixed in 3.7% formaldehyde in PBS, mounted under a no. 1 coverslip in glycerol−PBS (90:10), and examined using a Zeiss Axioplan epifluorescence microscope.

Competition Experiments. One hundred microliters of asialofetuin at 1 μg/mL was immobilized on Costar EIA plate wells by overnight incubation at 4 °C in PBS. Wells were blocked with 3% BSA and washed with PBS/0.1% Tween 20. One hundred microliter samples of plant RTB and various RTB mutants were diluted in EXCELL400 medium with or without 100 mM lactose or 100 μg/mL asialofetuin and added to wells. Twelve different concentrations of each protein were applied in each experiment. After 1 h at room temperature, wells were washed with PBS/0.1% Tween 20, reacted with rabbit anti-ricin (1:400 in PBS/0.5% BSA), rewashed and incubated with goat anti-rabbit Ig conjugated to alkaline phosphatase (Sigma; 1:5000 in PBS/0.5% BSA), washed again, and developed with *p*-nitrophenyl phosphate at 1 mg/mL in 50 mM diethanolamine, pH 9.6. Absorbance of wells was measured at 405 nm in a microtiter plate reader. Concentrations of protein giving half-maximal binding (K_d) in the presence or absence of competitor were calculated.

Reassociation of Mutant RTBs with Plant RTA To Form Heterodimers. Mutant RTBs (0.25 mL, 5−15 μg) were mixed with a 4-fold molar excess of plant RTA (Inland Laboratories) in 0.1 M triethylamine/sodium phosphate, pH 7, overnight at room temperature. The reaction mixture was then analyzed by a ricin ELISA utilizing P2 monoclonal anti-RTB coated wells and biotin conjugated αBR12 monoclonal anti-RTA and alkaline phosphatase conjugated strepavidin detection reagents as previously described (Frankel et al., 1996b).

Table 1: Insect-Derived RTB Proteins

protein	subdomains modified
RTB	
W37S/Y248H	1α, 2γ
W37S/Y248H/W160S	1α, 2α, 2γ
W37S/Y248H/Y78H	1α, 1β, 2γ

Sugar Binding of Mutant Heterodimers. Asialofetuin (1 μg/mL) was found to Costar EIA plate wells, and an ELISA was performed as previously detailed with samples of mutant RTB−RTA and castor bean ricin (Frankel et al., 1996b). Briefly, the asialofetuin-coated wells were washed with PBS plus 0.1% Tween 20, blocked with 3% BSA, rewashed and incubated with 12 different concentrations of samples in EXCELL400, rewashed and reacted with 100 μL of biotinylated αBR12 monoclonal anti-RTA antibody, rewashed and incubated with streptavidin−alkaline phosphatase, washed again, and developed with *p*-nitrophenyl phosphate in 50 mM diethanolamine, pH 9.6. Absorbance of wells was measured at 405 nm on a microtiter plate reader. Concentrations of protein giving half-maximal binding (K_d) were calculated.

Cytotoxicity of Recombinant Mutant Heterodimers. HUT102 cells were incubated with dilutions of ricin and mutant RTB−plant RTA heterodimers at varying concentrations for 24 h and pulsed for 4 h with [^{3}H]leucine as previously detailed (Frankel et al., 1996a). The IC_{50} for ricin and each mutant heterodimer was calculated as the concentration which inhibited protein synthesis by 50% compared with control.

RESULTS

Selection of Mutants. Our strategy to identify additional lectin binding subdomains in RTB was to perform mutational analysis on the double-site mutant RTB[W37S/Y248H]. This mutant had the same two log reduction in sugar binding avidity as observed with three other double-site mutants (RTB[D22E/D234E], RTB[K40M/N46G/N255G], and RTB-[D22Q/V23A/R24N/D234A/V235A/R236T]) (unpublished experiments). Importantly, the W37S/Y248H mutant was expressed and purified in yields similar to those of wild-type RTB. We reasoned that modification of an amino acid residue participating in sugar binding in an additional lectin site should produce further reduction in sugar binding avidity. The mutation must reduce sugar binding without altering protein folding to be significant. We used the available three-dimensional structure of ricin to identify subdomains 1β and 2α which had tertiary structure similar to the known lectin sites in subdomains 1α and 2γ. The aromatic amino acid residues projecting into the binding cleft of subdomain 1β and 2α are Tyr-78 and Trp-160, respectively. Substitutions at these positions which conserved the best side-chain size and polarity were histidine and serine, respectively. The list of mutants prepared is shown in Table 1.

Studies of Protein Integrity. Attributing a role in lectin function for individual amino acid residues by altered sugar binding after modification of the residue depends upon lack of participation of that residue in other functions such as

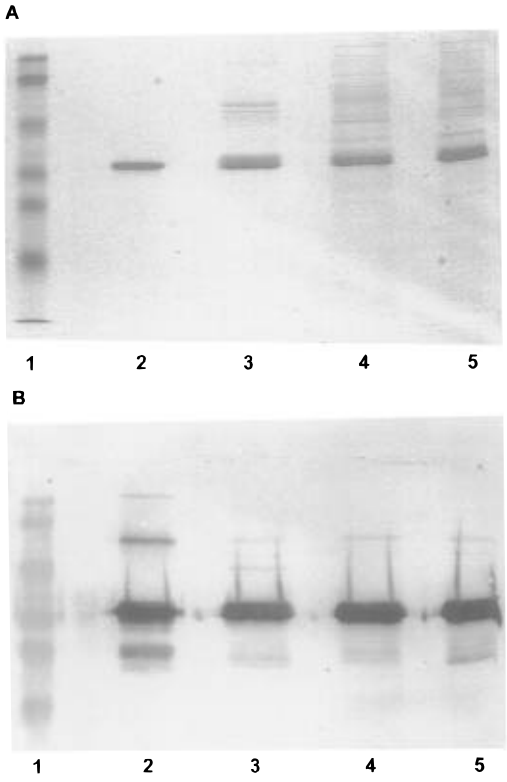

FIGURE 1: Insect-derived mutant RTBs. (A) Coomassie-stained 15% reducing SDS−PAGE: lane 1, low molecular mass prestained Bio-Rad protein standards; lane 2, plant RTB; lane 3, W37S/Y248H; lane 4, W37S/Y248H/W160S; lane 5, W37S/Y248H/Y78H. (B) Immunoblot using rabbit anti-ricin antibody of 15% reducing SDS−PAGE. Lanes are the same as in (A).

local folding. We indirectly tested protein folding integrity by measuring protein yield, immunoreactivity with a panel of monoclonal and polyclonal antibodies, CD spectra, protease sensitivity, and efficiency of reassociation with RTA. Protein yields are related to processing intracellularly in insect cells, avoidance of proteolytic degradation, and proper secretion.

Yields of mutant RTBs were estimated from the optical density at 280 nm of neutralized alkaline eluants, postaffinity 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 1A). Results were confirmed by densitometry of immunoblots reacted with rabbit anti-ricin antibody. As shown in Figure 1B, both triple-site mutants were reactive with the polyclonal antibody. Finally, a monoclonal antibody anti-RTB ELISA was used to verify concentrations of each mutant. All three assays gave similar values. The yield from cell supernatants of the parent double-site mutant, W37S/Y248H, was 205 μg/L Sf9 culture. The yield of the triple-site mutant, W37S/Y248H/Y78H, was 750 μg/L culture, and the yield of the triple-site mutant, W37S/Y248H/W160S, was 180 μg/L culture. Yields from cell extracts were similar to yields from supernatants for all three mutants.

Reactivities of the mutant RTBs with different monoclonal antibodies to RTB (P2, P8, and P10) were tested by substituting different monoclonal antibodies as capture reagents in the antibody ELISA. Equivalent results were observed for each antibody, further suggesting similar folding of the mutants. W37S/Y248H RTB reacted 1.3-fold and 4.0-fold more with P8 and P10 monoclonal antibodies relative to P2 antibody. W37S/Y248H/Y78 RTB reacted 0.7-fold and 1.8-fold as well with P8 and P10 antibodies as with P2 antibody. Finally, W37S/Y248H/W160S RTB bound 1.0-fold and 2.3-fold to P8 and P10, respectively, relative to P2.

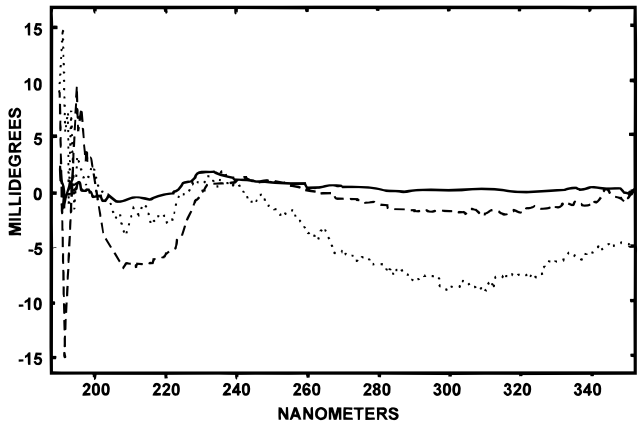

FIGURE 2: CD spectra of plant RTB and mutant RTBs. Summation of five spectra for each protein between 190 and 350 nm for (—) plant RTB, (···) 1α, 1β, 2γ triple-site mutant, and (- - -) 1α, 2α, 2γ triple-site mutant. Three point smoothing was used.

The CD spectra of the 1α, 1β, 2γ and 1α, 2α, 2γ mutants were similar to plant RTB. All showed peaks of 2 mdeg at 240 nm and troughs at 210 nm (Figure 2). The 210 nm trough was 1 mdeg for plant RTB, 5 mdeg for the 1α, 1β, 2γ mutant, and 6 mdeg for the 1α, 2α, 2γ mutant. The 1α, 1β, 2γ mutant also showed a trough at 300 nm of 8 mdeg versus 1 mdeg for plant RTB and no trough for the 1α, 2α, 2γ mutant. The 300 nm trough may have been due to protein contaminants.

Both plant RTB and insect-derived wild-type RTB were resistant to trypsin digestion with a 23 kDa fragment product observed only at a 1:1 enzyme:substrate ratio (Figure 3A). In contrast, single-site, double-site, and triple-site mutants were 100-fold more sensitive and displayed fragments at 23 and 15 kDa (Figure 3).

Since the intersubunit interface occupies 14% of the surface of RTB and multiple RTB residues participate in intersubunit bonds, optimal heterodimer formation requires intact RTB folding. Incubation of 5×10^{-7} to 2.5×10^{-6} M mutant RTBs with excess plant RTA overnight at room temperature led to 70% reassociation of W37S/Y248H, 24% reassociation of W37S/Y248H/Y78H, and 57% reassociation of W37S/Y248H/W160S. Similar levels of reassociation were seen using plant RTB or recombinant wild-type RTB with plant RTA under the same conditions. The heterodimer concentrations were quantitated by an ELISA which identified molecules with both RTB and RTA epitopes and by densitometry of 65 kDa bands of immunoblots with anti-RTB and anti-RTA antibodies (Figure 4). Both ELISA and immunoblots gave similar values and showed both mutants reassociated well with plant RTA and had minimal homodimer formation. Thus, reassociation data provide further evidence for proper folding of RTB mutants.

Mutant RTB Lectin Function. Lectin function was then assessed in mutants by multiple quantitative assays—RTB and heterodimer binding to immobilized asialofetuin, RTB binding to cells, competition of RTB binding to both immobilized asialofetuin and cells by soluble galactosides, and, finally, heterodimer cell cytotoxicity. Each assay gave matching results.

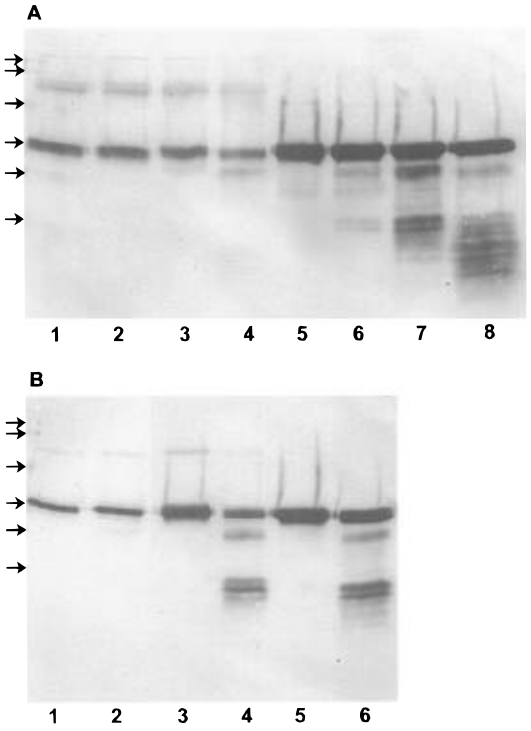

FIGURE 3: Trypsin sensitivity of plant RTB and mutant RTBs. Proteins were treated with trypsin as described in the text, and immunoblots were performed. Low molecular mass prestained Bio-Rad protein standards (101, 83, 50.6, 35.5, 29.1, 20.9 kDa) are indicated by arrows. (A) Lane 1, plant RTB without trypsin; lane 2, plant RTB with 1:100 trypsin; lane 3, plant RTB with 1:10 trypsin; lane 4, plant RTB with 1:1 trypsin. (B) Lane 1, plant RTB without trypsin; lane 2, plant RTB with 1:10 trypsin; lane 3, W37S/Y248H/W160S RTB without trypsin; lane 4, W37S/Y248H/W160S RTB with 1:10 trypsin; lane 5, W37S/Y248H/Y78H RTB without trypsin; lane 6, W37S/Y248H/Y78H RTB with 1:10 trypsin.

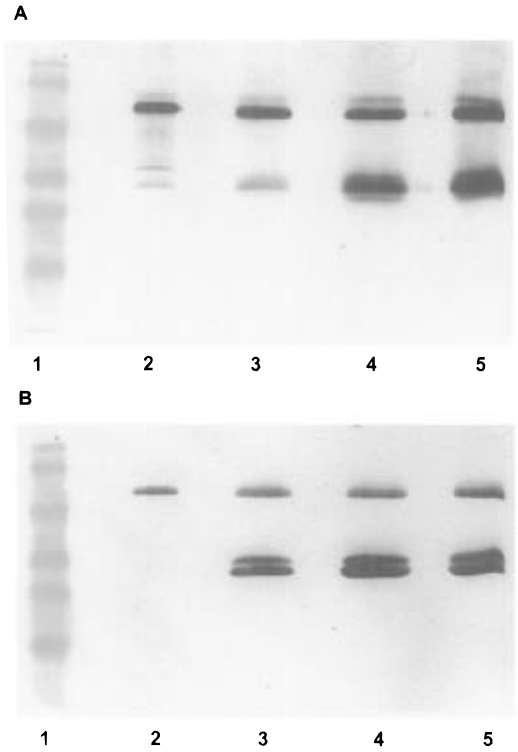

FIGURE 4: Reassociation of mutant RTBs with plant RTA. (A) Immunoblot of 15% nonreducing SDS−PAGE of reassociated mutant RTB−plant RTA: lane 1, low molecular mass Bio-Rad protein standards; lane 2, ricin; lane 3, W37S/Y248H-RTA; lane 4, W37S/Y248H/W160S RTA; lane 5, W37S/Y248H/Y78H RTA. The immunoblot uses monoclonal antibodies P2 and P10 anti-RTB. (B) Same as in (A) except the immunoblot uses monoclonal antibody αBR12 anti-RTA. The heterodimer appears at 60 kDa, and subunits appear at 30 kDa.

RTB mutant binding to immobilized asialofetuin was assayed first. The double-site RTB mutant, W37S/Y248H, bound asialofetuin 4.8 ± 2% ($n = 6$) relative to recombinant or plant RTB. The triple-site RTB mutant, W37S/Y248H/W160S, bound asialofetuin similarly−1.1 ± 0.27% ($n = 7$) relative to plant RTB. In contrast, the triple-site mutant, W37S/Y248H, showed minimal to neglible binding to asialofetuin at 0.2 ± 0.08% ($n = 7$) relative to plant RTB which was 2−3-fold higher than background.

Heterodimer binding to immobilized asialofetuin was next assayed. Ricin bound immobilized asialofetuin with a K_d of 4×10^{-9} M. In contrast, both RTB[W37S/Y248H]−RTA and RTB[W37S/Y248H/W160S]−RTA bound asialofetuin 1% as well as plant ricin with K_d's of approximately 4×10^{-7} M. RTB[W37S/Y248H/Y78H]−RTA bound 0.15% as well as plant ricin with a K_d of 6×10^{-6} M.

An independent measure of mutant RTB binding to glycoproteins was made by detecting mutant RTB bound to cell surfaces. Only W37S/Y248H and W37S/Y248H/W160S showed significant binding to KB cells at 4 °C (Figure 5).

Competition experiments were done next to determine if the observed binding was specific for galactosides. Binding of W37S/Y248H to immobilized asialofetuin was inhibited 3-fold by 100 mM α-lactose and 5-fold by 100 μg/mL asialofetuin. Similarly, W37S/Y248H/W160S binding was inhibited 9-fold by lactose and 27-fold by asialofetuin. Binding of the triple-site mutant W37S/Y248H/Y78H was inhibited 3-fold by either lactose or asialofetuin. The more marked inhibition of sugar binding by asialofetuin compared to lactose may be due to multivalent interactions with wild-type and mutant RTBs or altered geometry of association. Such a difference was observed for the double-site and 1α, 2α, 2γ triple-site mutant but not the 1α, 1β, 2γ triple-site mutant. Binding of wild-type RTB, the double-site mutant, and the 1α, 2α, 2γ triple-site mutant to KB cells was blocked by 100 μg/mL asialofetuin (Figure 5). No measurable binding of the 1α, 1β, 2γ triple-site mutant to KB cells was observed using the immunofluorescence assay, and hence, competition with this mutant could not be demonstrated.

Finally, mammalian cell cytotoxicity of the mutant heterodimers was tested. The IC_{50} of ricin on HUT102 human leukemia cell was 4×10^{-12} M (Figure 6). The IC_{50} for W37S/Y248H was 2×10^{-10} M; the IC_{50} for W37S/Y248H/W160S was 1×10^{-10} M; and the IC_{50} for W37S/Y248H/Y78H was 5×10^{-9} M. Plant RTA alone had a 20-fold higher IC_{50} of 10^{-7} M. These results show a 25-fold reduction in toxicity for the 1α, 1β, 2γ mutant versus no reduction in toxicity for the 1α, 2α, 2γ mutant compared to the parent double-site mutant. RTA was less toxic by an additional 20-fold relative to the 1α, 1β, 2γ mutant, suggesting either incomplete inactivation of one of the three sites or an additional unidentified fourth site.

DISCUSSION

RTB Structural Modifications. Examination of the tripeptide kink regions of subdomains 1β and 2α revealed the protruding side chain of aromatic ring residues Tyr-78 for

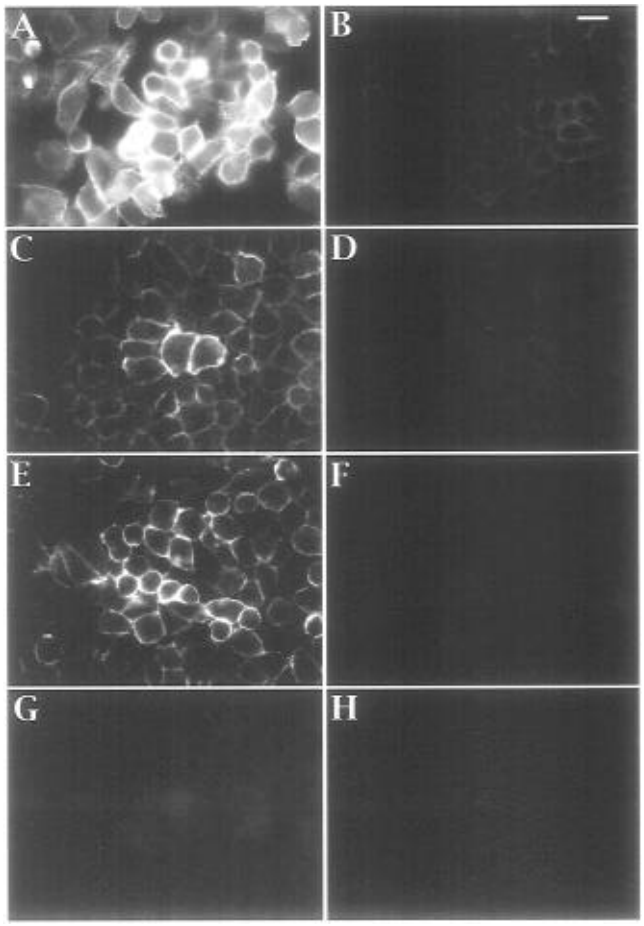

FIGURE 5: Binding of mutant RTBs to KB cells. Cells were treated and examined as previously described (12−14). Magnification = 250×; bar = 20 μm. (A, C, E, G) Without 100 μg/mL asialofetuin; (B, D, F, H) with asialofetuin. (A, B) Plant RTB; (C, D) W37S/Y248H; (E, F) W37S/Y248H/W160S; (G, H) W37S/Y248H/Y78H.

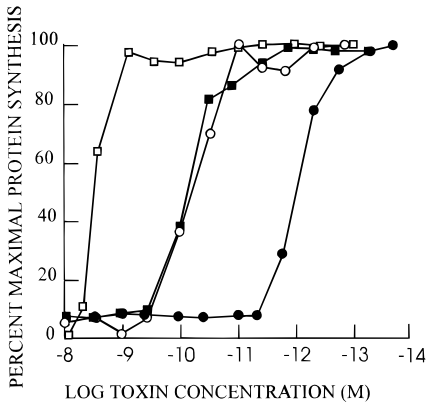

FIGURE 6: HUT102 cell cytotoxicity. Assay is as described in the text. Symbols: (●) ricin $IC_{50} = 1 \times 10^{-12}$ M; (○) W37S/Y248H-RTA $IC_{50} = 2 \times 10^{-10}$ M; (■) W37S/Y248H/W160S-RTA $IC_{50} = 1 \times 10^{-10}$ M; (□) W37S/Y248H/Y78H-RTA $IC_{50} = 5 \times 10^{-9}$ M. The yield of reassociated heterodimer was tested by modified ELISA. Each experiment was performed in triplicate.

subdomain 1α and less optimal exposure of Trp-160 from subdomain 2α (Rutenber & Robertus, 1991). Vyas reviewed the major features of protein−carbohydrate interactions based on X-ray crystallography, and the stacking of aromatic residues against the face of sugars was seen in all but one known protein−carbohydrate complex (Vyas, 1991). The nonpolar face of the pyranose ring apposes the nonpolar aromatic side chain with van der Waals forces. On the basis of similar subdomain folding, we reasoned that Tyr-78 and Trp-160 may stabilize galactosides similar to Trp-37 and Tyr-248 of subdomains 1α and 2γ, respectively (Rutenber & Robertus, 1991). We further hypothesized that modification of single amino acid residues in each subdomain should provide maximal lectin-site alteration with minimal disruption of protein secondary and tertiary structure. However, residual sugar binding may exist in the various sites even after single amino acid mutations. Further, mutations in one site may subtly alter protein folding and lead to changes in remote lectin sites. Nevertheless, even in the absence of corroborating crystallographic structures, mutational analysis may provide provocative evidence for the location and relative roles of protein functional domains.

Folding of RTB Mutants. Folding of the double-site and triple-site mutants was assessed by purification yields, immunoreactivities with a panel of polyclonal and monoclonal antibodies, CD spectra, protease sensitivity, and ability to reassociate with plant RTA. On the basis of similarities of each of these characteristics with wild-type insect-derived RTB, we propose similar local folding in the mutants.

The recovery of 0.18 and 0.75 mg of triple-site mutant RTB/L infected Sf9 cell supernatants was higher than the yields of six single-site RTB mutants and four double-site RTB mutants (Frankel et al., 1996a,b; unpublished experiments). Further, similar quantities of triple-site mutants were recovered from cell pellets−440 μg/L for W37S/Y248H/Y78H RTB and 70 μg/L for W37S/Y248H/W160S RTB. The yields were not dissimilar from the yields for the double-site mutant W37S/Y248H (220 μg/L from supernatant and 250 μg/L from cell pellet) or wild-type RTB (400 μg/L for supernatants) in this expression system and may reflect proper folding for the triple-site mutants. The conservative modification of surface residues (Trp to serine and Tyr to histidine) may have contributed to protein stability.

Both triple-site mutants reacted with all monoclonal and polyclonal anti-RTB antibodies tested. Further evidence that these RTB mutants were properly folded included their stability at 4 and −20 °C for 3 months in 0.1 M triethylamine/sodium phosphate, pH 8.0, their ability to reassociate with plant RTA, and their CD spectra. There was greater sensitivity of the mutant RTBs to trypsin, suggesting subtle differences in folding. But no differences were observed among the different mutants, although they varied greatly in lectin function.

Sugar Binding of Triple-Site Mutants. W37S/Y248H/W160S RTB retained binding to immobilized asialofetuin and KB cell surface glycoproteins. In both cases, the binding was competed with soluble saccharides. In contrast, W37S/Y248H/Y78H had minimal sugar binding. RTB mutants reassociated with RTA retained similar lectin activity, suggesting that the three sites are accessible in both the free subunit and holotoxin. Our results differ from the findings of Yen and Vitetta with Cos cell-derived mutant RTB (Vitetta & Yen, 1990), Wales and colleagues with *Xenopus laevis* cell-derived RTBs (Wales et al., 1991), and Swimmer and colleagues with bacteriophage gene III fusion proteins (Swimmer et al., 1992). They reported complete inactivation of sugar binding by modifications of residues in a single subdomain (Vitetta & Yen, 1990) or two subdomains (Wales et al., 1991; Swimmer et al., 1992). However, very small amounts of protein were made, and no purification or immunological characterization of the products was done.

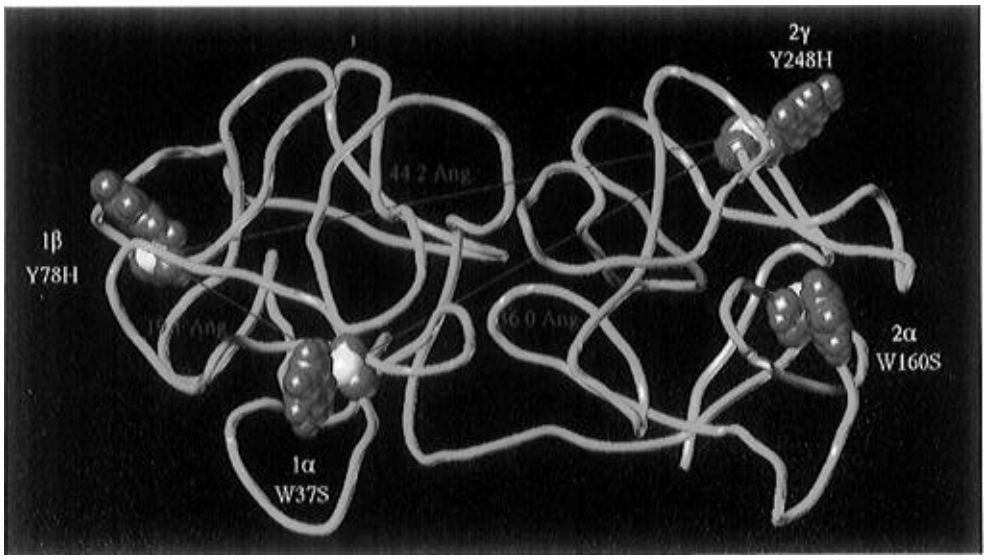

FIGURE 7: Model of RTB showing the α-carbon backbone as a blue tube, sugar binding sites identified by lactose molecules, and intersite distances in red. The structure is based on Brookhaven coordinates derived from Rutenber and Robertus (1991) using SYBYL software on a Silicon Graphics Iris Indigo workstation.

In each case, decreased sugar binding may have been due, in part, to misfolding or aggregation of recombinant RTBs, leading to an overestimation of the effect of their modifications.

The small residual binding of the 1α, 1*β*, 2*γ* triple mutant may be due to incomplete inactivation of one or more sites. One possible source is subdomain 1α where W37S leaves 24% binding relative to wild type versus 12−15% for several other 1α subdomain mutants (K40M and K40M/N46G). Alternatively, RTB possesses a fourth undefined lectin site.

Cell Intoxication Functions of the Triple-Site Mutants. Cell sensitivity to mutant heterodimers paralleled mutant residual sugar-binding activity. Significant residual potency was seen with the 1α, 2*γ* and 1α, 2α, 2*γ* mutant heterodimers. Their IC_{50}'s were at least two and one-half logs lower than plant RTA alone (RTB alone was nontoxic with $IC_{50} > 6 \times 10^{-6}$ M). In contrast, the 1α, 1*β*, 2*γ* mutant RTB−RTA had minimal toxicity above background. Since we have demonstrated potent ($IC_{50} = 1 \times 10^{-12}$ M) killing of receptor-positive cells by IL2-W37S/Y248H RTB−RTA (unpublished experiments), it will be of interest to examine the potency of IL2-triple-site mutant RTB−RTA fusion molecules on receptor-positive cells. If intracellular sugar binding is required for cell intoxication, as reported by several groups (Goldmacher et al., 1992; Newton et al., 1992), the latter molecule should be nontoxic.

Widely Separated Ricin Lectin Sites. The 1α and 2*γ* sites are separated by 36 Å (Figure 7). 1*β* and 2*γ* sites are 44 Å apart. The 1α and 1*β* distance is 19 Å. These interbinding site distances are much larger than the intersite spacing for the hepatic Gal/GalNac receptor and its triantennary *N*-glycoside ligand (10−20 Å) (Lee, 1992). Instead, the ricin geometry resembles the spacing of sites on surface-binding lectins including mammalian mannose-binding protein, influenza virus hemagglutinin, pertussis toxin, and cholera toxin (Drickamer, 1995). These proteins are phylogenetically unrelated, based on lack of primary or tertiary structure homology. Nevertheless, in all these proteins, the sugar-combining sites are multiple widely spaced and project toward a single plane. Thus, they are ideally suited for binding to eukaryotic cell surfaces.

Three RTB Lectin Sites. The three binding sites on ricin may provide the optimal geometry for binding to the uneven galactosyl oligosaccharide-rich surface of mammalian cells similar to camera tripods or stools. The RTB 1*γ* and 2*β* subdomains are unlikely to contribute additional sugar binding as they lack the tripeptide α-carbon kink, aromatic residues, or charged residues for hydrogen bond formation. Further, no RGD-like domains exist in RTB—unlike discoidin I from the slime mold *Dictylostelium discoideum* (Poole et al., 1981).

Biochemical modification studies provide additional evidence for three RTB lectin sites. *N*-Acetylimidazole O-acetylated two tyrosines on ricin leading to reduced sugar binding (Youle et al., 1981), implicating sites in the 2*γ* and, perhaps, 1*β* pockets. In addition, *N*-bromosuccinimide modified one tryptophan (Trp-37) reducing sugar binding, demonstrating a sugar-binding site in the 1α subdomain fold (Hatakeyama et al., 1986). In a different approach, Lambert cross-linked three distinct sites on ricin with the radiolabeled activated 6-(*N*-methylamino)-6-deoxy-D-galactose moiety (Lambert et al., 1991). Sequential loss of sugar binding and cell intoxication was observed with one, two, or three cross-linkers per ricin molecule (Goldmacher et al., 1992).

Targeted Ricin Molecules for Clinical Development. A variety of peptide toxins have been linked to monoclonal antibodies and growth factors and used for targeted cell depletion in vitro, in animal models, and in patients with cancer and autoimmune diseases (Frankel et al., 1995). A critical step in the synthesis of such targeted toxins is the modification or removal of normal tissue-binding domains. The existence of a third lectin site on ricin may explain some of the toxicities observed with doubly blocked ricin immunoconjugates (Grossbard et al., 1992). Engineering of either modified ricin fusion molecules or immunoconjugates in the future should include modification of all three ricin sites for optimal patient safety. The identification of the third galactose-binding site on subdomain 1*β* should facilitate construction of such therapeutics.

ACKNOWLEDGMENT

We thank Starr Hazard and James Nicholson for molecular graphics and imaging analysis, respectively. We appreciate the outstanding technical assistance of Josef Vesely and Billie Harris. We also thank Drs. Walter Blattler and Jerry Fulton for providing monoclonal antibodies to ricin and ricin subunits, respectively. We thank Dr. Kevin Shea, Department of Pharmacology, MUSC, and the MUSC Shared Spectroscopy Facility for acquisition of CD spectra.

REFERENCES

Baenziger, J., & Fiete, D. (1979) *J. Biol. Chem. 254*, 9795.
Drickamer, K. (1995) *Struct. Biol. 2*, 437.
Endo, Y., & Tsurugi, K. (1987) *J. Biol. Chem. 262*, 8128.
Frankel, A., Tagge, E., & Willingham, M. (1995) *Semin. Cancer Biol. 6*, 307.
Frankel, A., Tagge, E., Chandler, J., Burbage, C., Hancock, G., Vesely, J., & Willingham, M. (1996a) *Bioconjugate Chem. 7*, 30.
Frankel, A., Tagge, E., Chandler, J., Burbage, C., & Willingham, M. (1996b) *Protein Eng.* (in press).
Goldmacher, V., Lambert, J., & Blattler, W. (1992) *Biochem. Biophys. Res. Commun. 183*, 758.
Grossbard, M., Lambert, J., Goldmacher, V., Blattler, W., & Nadler, L. (1992) *Cancer Res. 52*, 4200.
Hatekeyama, T., Yamasaki, N., & Funatsu, G. (1986) *J. Biochem. 100*, 781.
Houston, L., & Dooley, L. (1982) *J. Biol. Chem. 257*, 4147.
Lambert, J., McIntyre, G., Gauthier, M., Zullo, D., Rao, V., Steeves, R., Goldmacher, V., & Blattler, W. (1991) *Biochemistry 30*, 3234.
Lee, Y. (1992) *FASEB J. 6*, 3193.
Lewis, M., & Youle, R. (1986) *J. Biol. Chem. 261*, 11571.
Newton, D., Wales, R., Richardson, P., Walbridge, S., Saxena, S., Ackerman, E., Roberts, L., Lord, J., & Youle, R. (1992) *J. Biol. Chem. 267*, 11917.
Poole, S., Firtel, R., Lamar, E., & Rowekamp, W. (1981) *J. Mol. Biol. 153*, 273.
Rivera-Sagredo, A., Solis, D., Diaz-Maurino, T., Jimenez-Barbero, J., & Martin-Lomas, M. (1991) *Eur. J. Biochem. 197*, 217.
Rutenber, E., & Robertus, J. (1991) *Proteins 10*, 260.
Sandvig, K., & Olsnes, S. (1982) *J. Biol. Chem. 257*, 7504.
Sandvig, K., Olsnes, S., & Pihl, A. (1976) *J. Biol. Chem. 251*, 3977.
Swimmer, C., Lehar, S., McCafferty, J., Chiswell, D., Blattler, W., & Guild, B. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 3756.
van Deurs, B., Petersen, O., Olsnes, S., & Sandvig, K. (1987) *Exp. Cell Res. 171*, 137.
Vitetta, E., & Yen, N. (1990) *Biochim. Biophys. Acta 1049*, 151.
Vyas, N. (1991) *Curr. Opin. Struct. Biol. 1*, 732.
Wales, R., Richardson, P., Roberts, L., Woodland, H., & Lord, J. (1991) *J. Biol. Chem. 266*, 19172.
Wales, R., Roberts, L., & Lord, J. (1993) *J. Biol. Chem. 268*, 23986.
Youle, R., Murray, J., & Neville, D. (1981) *Cell 23*, 551.
Zentz, C., Frenoy, J., & Bourrillon, R. (1978) *Biochim. Biophys. Acta 536*, 18.

BI960798S