Molecular, Biological, and Preliminary Structural Analysis of Recombinant Bryodin 1, a Ribosome-Inactivating Protein from the Plant *Bryonia dioica*

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ABSTRACT: *Bryonia dioica* (*Cucurbitaceae* family) produces at least two type I ribosome-inactivating proteins, bryodin 1 (BD1) and bryodin 2 (BD2). A cDNA sequence encoding BD1 was isolated from *B. dioica* leaf mRNA using degenerative oligonucleotides and codes for a 22 amino acid signal peptide followed by a protein of 267 residues. Expression of two recombinant BD1 (rBD1) forms in *Escherichia coli* yielded proteins of 267 (to the natural stop codon) and 247 amino acids (to the putative cleavage site yielding the mature protein) that had identical protein synthesis inhibition activity as compared to native BD1. The substitution of Lys for Glu at position 189 near the active site reduced the ability of rBD1 to inhibit protein synthesis by 10-fold. Toxicologic analysis showed that rBD1 was well tolerated in rodents with LD_{50} values of 40 mg/kg in mice and >25 mg/kg in rats. A crystal of mature rBD1 protein was used to collect X-ray diffraction data to 2.1 Å resolution. The rBD1 crystal structure was solved and showed extensive homology with other type I RIPs and A chains of type II RIPs. The studies described here demonstrate that rBD1 retains full biologic activity and serve as a guide for using this potent, yet nontoxic, RIP in the construction of single-chain immunotoxin fusion proteins.

The isolation and characterization of new ribosome-inactivating proteins (RIPs)[1] that have low toxicity to animals but are potent once inside target cells are important for the development of improved immunotoxins for the treatment of disease. Roots and leaves of *Bryonia dioica*, a member of the plant family *Cucurbitaceae*, produce at least two RIPs, bryodin 1 (BD1) and bryodin 2 (BD2), which are 29 and 27 kDa in mass, respectively (Stirpe et al., 1986; Siegall et al., 1994a). Native BD1 has low toxicity in mice (LD_{50} >40 mg/kg) and is potent in inhibiting protein synthesis (EC_{50} = 7 pM) in a cell-free rabbit reticulocyte lysate assay (Siegall et al., 1994a). Due to these properties, BD1 represents a potential improvement as a component of immunotoxins over other commonly used RIPs such as ricin A chain, pokeweed antiviral protein, and saporin, which are quite toxic to mice with LD_{50}'s of 5, 2.5, and 4 mg/kg, respectively (Siegall et al., 1994a; Battelli et al., 1990). Binding defective bacterial toxins that have been used in immunotoxin construction, such as PE40 from *Pseudomonas* exotoxin A, are also toxic to mice (LD_{50} value of 2.5 mg/kg) (Hwang et al., 1987; Kondo et al., 1988).

As is the case for other RIPs including trichosanthin (Chow et al., 1990; Shaw et al., 1994), saporin (Barthelemy et al., 1993; Barbieri et al., 1993), and pokeweed antiviral protein (Irvin & Uckun, 1992), bryodins belong to a multigene family. Plant RIPs have been placed into two groups on the basis of their structure (Barbieri et al., 1993). Type I RIPs including bryodin, gelonin, saporin, and trichosanthin contain a single chain that has enzymatic activity but no binding domain. Type II RIPs such as ricin and abrin contain two chains, an A chain that is catalytically active and is structurally similar to type I RIPs and a B chain that contains a cell binding domain with lectin properties. Type II RIPs are thereby cytotoxic to many cell types. Both types of RIPs inhibit protein synthesis by inactivating the 60S subunit of eukaryotic ribosomes through cleavage of the *N*-glycosidic bond of adenine 4324 in 28S rRNA (Endo et al., 1987).

Because of BD1's low animal toxicity, an immunotoxin conjugate of BD1 and mAb BR96 was constructed. The immunoconjugate BR96−BD1 was found to be specifically cytotoxic to BR96 antigen positive cells while not affecting cells that did not express the antigen (Siegall et al., 1994a). Since single-chain immunotoxins have been shown to be more effective as antitumor agents than similarly composed chemically conjugated immunotoxins (Chaudhary et al., 1989; Friedman et al., 1993; Brinkmann & Pastan, 1994), it was of interest to isolate a cDNA encoding BD1 for future use as a component of a fusion toxin.

In this study we report the cloning and full-length sequence of a cDNA encoding BD1. From the deduced amino acid sequence of the protein, a putative cleavage site 20 amino acids from the carboxyl terminus, which may be involved in the production of mature BD1, has been identified. Expression vectors were engineered encoding two recombinant BD1 (rBD1) forms, one extending to the natural stop codon and a second to the potential cleavage site. Following expression in *Escherichia coli* as intracellular inclusion bodies, the rBD1 forms were denatured, refolded, and compared to native BD1 in both cell-free and cell-based protein synthesis inhibition assays. A mutant form of BD1

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[1] Abbreviations: RIP, ribosome-inactivating protein; BD1, bryodin 1; ELISA, enzyme-linked immunosorbent assay; RACE, rapid amplification of cDNA ends; rBD1, recombinant BD1.

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which introduced a single point mutation near the putative active site as predicted by sequence similarities with other RIPs was generated and tested for catalytic and biological activity. Additionally, the LD_{50} value of mature rBD1 was determined in both mice and rats and contrasted with that of ricin A chain and PE40. Lastly, rBD1 was crystallized and its structure was determined by X-ray crystallography. On the basis of the preliminary crystallographic results, an analysis of structure−function relationships has been initiated.

EXPERIMENTAL PROCEDURES

Reagents. *B. dioica* roots were purchased from Poyntzfield Herb Nursery in Black Isle by Dingwall, Rossshire, U.K., and grown in our laboratory in order to obtain leaves for RNA isolation. [^{3}H]Leucine and [^{32}P]dATP were purchased from New England Nuclear (Boston, MA). ABC immunoblot kits were purchased from Vector Laboratories (Burlingame, CA). Oligonucleotides were synthesized on a 394 automatic DNA/RNA synthesizer from Applied Biosystems (Foster City, CA) using 0.2 μmol of scale B cyanoethyl phosphoramidites. Deglycosylated ricin A chain was purchased from Inland Laboratories (Austin, TX). PE40 was produced as previously described (Debinski et al., 1992).

RNA Preparation. Total RNA was extracted from *B. dioica* leaf material (2.1 g) using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's protocol. The resulting total RNA was quantitated and analyzed by electrophoresis in formaldehyde−agarose gels and visualized by staining with ethidium bromide. The approximate yield was 0.9 mg of total RNA/g of leaf material.

PCR Amplification of cDNA for BD1. PCR reactions containing DNA or RNA template (1 μg), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 25 pM primers, and 2.5 units of Taq I DNA polymerase (Perkin-Elmer Cetus) were incubated at 94 °C for 3 min and 25 cycles at 94 °C, 15 s/55 °C, 15 s/72 °C, 1.25 min, and 6 min at 72 °C. The PCR products were analyzed by 1.2% agarose gel electrophoresis.

Oligo(dT)-primed cDNA (using total RNA as template) was PCR amplified using degenerate oligonucleotide mixtures of 5′-CCTAGCCCATGGATGT[T/G]AGCTT [C/T]-CGTTT-3′ and 5′-CCTAGCGAATTCCTA[C/G]AGAGG-[G/T]AT[G/A]TTGT A[G/C]AC corresponding to residues 1−6 and 31−36, respectively, to isolate an exact BD1 sequence between amino acids 7 and 30 (degenerate positions indicated by brackets). The products of the PCR reaction were subcloned into the vector pCR II as per manufacturer's protocol using the TA cloning kit (Invitrogen Corp., San Diego, CA). Recombinant clones were identified by PCR using universal forward and reverse primers that encompass the inserted DNA and sequencing of inserts that were 100−120 bp in size. DNA sequencing was performed by using Sequenase, Version 2 (United States Biochemical Corp., Cleveland, OH) as per manufacturer's instructions. One clone containing the correct nucleotide match to the amino-terminal sequence of BD1 was identified following sequence analysis of 81 individual clones.

The 3′ end of the BD1 gene was amplified using the 3′ RACE System (Gibco BRL, Gaithersburg, MD) as per manufacturer's protocol. Briefly, following first strand cDNA synthesis from total RNA, the 3′ end of BD1 was PCR amplified using oligonucleotide primers 5′-TTATCAG-GTGCTACAACCACATCC-3′, corresponding to residues 6−13, and 5′-GACTCGAGTCGACATCG-3′. The PCR product was cloned into pCR II, and clones containing BD1 sequences were selected by hybridization on nylon filters probed with ^{32}P-labeled exact oligonucleotide probe that hybridized to codons 23−29. Two clones that encode the BD1 gene were identified by DNA sequencing. To identify the exact 5′ end of the BD1 gene, 5′ RACE was performed using the 5′ RACE System (Gibco BRL) as per manufacturer's protocol. Briefly, first-strand cDNA was synthesized using a primer corresponding to residues 251−267, tailed with dCTP, and PCR amplified with oligonucleotide primers 5′-GAGAGGTATGTTGTAGACTTTCCT-3′, corresponding to residues 29−36, and 5′-GGCCACGCGTCGACTAG-TACGGGIIGGGIIGGGIIG. The resulting DNA fragments were subcloned into pCR II as before. BD1-containing clones were selected by hybridization of filters probed with a ^{32}P-labeled oligonucleotide corresponding to residues 6−13. Two positive BD1-containing cDNA clones were identified by DNA sequencing.[2]

Construction of Expression Plasmids Encoding rBD1. The BD1 cDNA sequence was used to PCR-amplify an 843 bp DNA fragment with primers corresponding to residues 1−13 and 251−267. The 5′ PCR primer was designed to encode a unique *Nco*I restriction site adjacent to an ATG translational initiation codon and the first 13 codons of the BD1 gene. The 3′ PCR primer was designed to anneal to the last 17 codons of the BD1 gene corresponding to the carboxyl terminus of the BD1 protein ending at the natural stop codon. The 3′ primer also contains two consecutive stop codons followed by an *Eco*RI restriction site. After PCR amplification and digestion with *Nco*I and *Eco*RI, the 810 bp *Nco*I−*Eco*RI fragment was ligated into a 5465 bp *Nco*I−*Eco*RI vector fragment prepared from plasmid pET22b (Novagen, Madison, WI). The product of this ligation was an expression vector designated pSE 12.0 containing the T7 promoter, the pelB leader sequence, and the cDNA sequence encoding BD1 to amino acid 267. Due to the difficulty in producing rBD1 protein with the pelB leader sequence (data not shown), subsequent cloning was performed to remove this sequence. Digestion of pSE 12.0 with *Xba*I and *Nco*I to remove the pelB leader sequence, followed by ligation with an oligonucleotide duplex restoring the non-pelB-containing sequences resulted in expression plasmid pSE 14.0 encoding $rBD1^{267}$.

A second rBD1 expression construct was prepared in which sequences encoding amino acids 248−267 of BD1 gene were deleted. Plasmid pSE 13.0, encoding BD1 residues 1−247 ($rBD1^{247}$), was constructed similarly to pSE 14.0, except that a primer corresponding to residues 239−247 was used in place of the primer corresponding to residues 251−267 for amplification of a 770 bp DNA fragment.

Purification of Recombinant BD1. The two BD1 expression plasmids, pSE 13.0 and pSE 14.0, were transformed into BL21(λDE3) and cultured until the cells reached an OD_{650} of 0.8−1.2 and induced with 1 mM IPTG for 90 min. Cultures were harvested by centrifugation, resuspended in TE sucrose (50 mM Tris-HCl and 1 mM EDTA, pH 8.0,

[2] The nucleotide sequence reported in this paper has been deposited in the GenBank/EMBL Data Bank with accession number L42298.

20% sucrose), and kept on ice for 15 min. Following centrifugation the pellet was osmotically shocked with ice-cold water, centrifuged, and resuspended in TE containing 0.25 mg/ml lysozyme. Tergitol (4% final volume) was added to the lysed cell solution, which was incubated at 4 °C for 30 min and centrifuged. The resulting inclusion bodies were washed 3 times with TE, resuspended in 10 mL of guanidine solution (7 M guanidine hydrochloride, 100 mM Tris-HCl, pH 8.0, 5 mM EDTA), sonicated (3 × 30 s), incubated on ice (1−2 h), and then centrifuged. The denatured protein in the supernatant was rapidly refolded in phosphate-buffered saline containing 0.4 M L-arginine and 70 mM guanidine solution at a concentration of <100 mg/L. The refolded protein was dialyzed against 10 mM NaH_2PO_4, pH 6.5, and loaded onto a CM-Sepharose column (Pharmacia, Uppsala, Sweden). Recombinant BD1 was eluted with a 0−0.3 M NaCl gradient in 10 mM NaH_2PO_4, pH 6.5. The fractions were analyzed by Coomassie-stained SDS−PAGE and immunoblots stained with polyclonal anti-BD antiserum.

Construction of Mutant BD1 Form. A single point mutant in the BD1 gene was generated using the Transformer site-directed mutagenesis kit (Clontech, Palo Alto, CA) as per manufacturer's protocols. Briefly, pSE 13.0 was mutated using the selection primer, which changes the *Pvu*I restriction site in the vector to *Nde*I, and with a primer which changed Glu^{189} (GAA) to Lys^{189} (AAG) in the BD1 cDNA. DNA from colonies was screened by restriction enzyme digestion and sequenced for authenticity. Recombinant BD1 Lys^{189} protein was expressed in *E. coli* and purified as previously described.

Cell-Free Protein Synthesis Inhibition Assay. Inhibition of protein synthesis was determined using a cell-free rabbit reticulocyte lysate translation system (Promega Biotec, Madison, WI) as previously described (Siegall et al., 1994a). RIPs were mixed in a volume of 25 μL with rabbit reticulocyte lysate (70% of reaction volume), a mixture of all amino acids (minus leucine) at 1 mM, 0.5 mCi/mL [^{3}H]-leucine, and brome mosaic virus RNA as substrate (0.5 μg). The reaction was incubated at 30 °C for 1 h and terminated by addition of 1 M NaOH and 2% H_2O_2. The translation product was precipitated with ice-cold 25% trichloroacetic acid (TCA) and 2% casamino acids as carrier for 30 min on ice. The radiolabeled proteins were harvested on glass fiber filters, rinsed with 5% TCA, rinsed with ethanol, dried, and quantitated using a scintillation counter.

Cell-Based Protein Synthesis Inhibition Assay. Human placental choriocarcinoma (JAR) cells were purchased from ATCC and cultured in RPMI 1640 supplemented with 10% FBS and 1% penicillin/streptomycin. Inhibition of protein synthesis by BD1 (native and recombinant forms) was measured in 10^5 cells/ml, 16 h after plating. Dilutions of BD1 forms were made in leucine-free RPMI, and 0.1 mL was added to each well (in 96 well format) for 20 h at 37 °C. Each dilution was done in quadruplicate. The cells were pulsed with [^{3}H]leucine (1 μCi/well) for an additional 4 h at 37 °C, lysed by freeze-thawing, and harvested using a Tomtec cell harvester (Orange, CT). Incorporation of [^{3}H]-leucine into cellular protein was determined using an LKB Beta-Plate liquid scintillation counter.

Toxicity of Protein Toxins in Mice and Rats. Mice (6−8 week old female BALB/c) and rats (6−8 week old female Wistar Furth) were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Toxicity of rBD1 in mice and rats, deglycosylated ricin A chain (dgRA) in rats, and PE40 in mice was determined by intravenous administration of purified proteins diluted in PBS and intravenous administration of up to 40 mg/kg for rBD1 in mice, 25 mg/kg for rBD1 in rats, 10 mg/kg for dgRA in rats, and 5 mg/kg for PE40 in mice. Animals were monitored for at least 7 days following injection.

Crystallization and Preliminary Structure Determination of rBD1^{247}. Orthorhombic crystals of rBD1^{247} were grown from buffered solutions of poly(ethylene glycol) 5000 monomethyl ether at pH 6. X-ray diffraction data (Cu Kα) were collected on a Molecular Structure Corp. R-axis IIC image plate detector mounted on a Rigaku RU300 generator run at 50 kV and 100 mA. Diffraction data 90% complete to 2.1 Å were collected from a crystal soaked in stabilizing solution with 15% (v/v) glycerol for 1 min and then flash-cooled to 100 K. The overall agreement between symmetry-related intensities is 4.3%. The space group is $P2_12_12_1$ (a = 65.5 Å, b = 77.4 Å, and c = 115.1 Å) with two molecules in the asymmetric unit. The structure was solved by the molecular replacement program AMoRe (Navaza, 1994) with the trichosanthin-based (PDB entry 1MRJ)[3] search model (Bernstein et al., 1987). The molecular replacement solution was refined with the program X-PLOR (Brunger et al., 1987) based on the intensity data from 8.0 to 2.1 Å resolution with signal-to-noise ratios of >1.[4] Electron density for all 247 residues plus an N-terminal Met is seen for both molecules. The current model includes 247 of the 248 residues (all but the N-terminal methionine) for each molecule, isotropic atomic temperature factors, and no solvent. Refinement without noncrystallographic restraints, which were eliminated because of small, but persistent differences between the two unique molecules, gives an overall average difference between observed intensities and those calculated on the basis of the model of 24% at this stage. Electron density is observed in the current map for both of the N-terminal methionines and for a number of potential ordered solvent molecules. Further model building and refinement are in progress.

RESULTS

Isolation of cDNA Sequence Encoding BD1. The cDNA sequence encoding BD1 was isolated in three steps: PCR amplification of an 111 bp DNA fragment from the 5′ end of the BD1 gene, 3′ RACE through the polyadenylation site of BD1, and 5′ RACE through the amino-terminal signal peptide of BD1. Degenerate oligonucleotides corresponding to residues 1−7 and 31−36 of BD1 (Siegall et al., 1994a) were used as primers for PCR amplification of reverse transcribed total RNA isolated from *B. dioica*. A 111 bp sequence that matched the N-terminal amino acid residues of BD1 was isolated and sequenced, allowing for the selection of exact oligonucleotide primers for 3′ and 5′ RACE, as described in Experimental Procedures. Two cDNA clones encoding BD1 were obtained from 3′ RACE and differed only in their 3′ noncoding region in that they

[3] Protein Data Bank entry 1MRJ (1994), 1.6 Å resolution structure of the complex of trichosanthin with adenine, Q. Huang, S. Liu, Y. Tang, S. Jin, and Y. Wang.

[4] Protein Data Bank entry 1BRY (1997), preliminary 2.1 Å coordinates for rBD1.

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                                                                    TCATCGAAATTCATCAAGACTGAATTGAAAGAGTAAAAAAAAA      43

-23         -20                                        -10                                          1
Met Ile Lys Leu Leu Val Leu Trp Leu Leu Ile Leu Thr Ile Phe Leu Lys Ser Pro Thr Val Glu Gly Asp Val
ATG ATC AAA TTG TTA GTC CTT TGG TTG CTA ATT CTC ACC ATA TTC CTC AAA TCT CCA ACT GTT GAG GGC GAT GTT     118

                            10                                         20
Ser Phe Arg Leu Ser Gly Ala Thr Thr Thr Ser Tyr Gly Val Phe Ile Lys Asn Leu Arg Glu Ala Leu Pro Tyr
AGC TTC CGT TTA TCA GGT GCT ACA ACC ACA TCC TAT GGA GTT TTC ATT AAA AAT CTG AGA GAA GCT CTT CCA TAC     193

                            35                                         45
Glu Arg Lys Val Tyr Asn Ile Pro Leu Leu Arg Ser Ser Ile Ser Gly Ser Gly Arg Tyr Thr Leu Leu His Leu
GAA AGG AAA GTG TAC AAT ATA CCG CTA TTA CGT TCA AGT ATT TCA GGT TCA GGA CGC TAC ACA TTA CTC CAT CTC     268

                            60                                         70
Thr Asn Tyr Ala Asp Glu Thr Ile Ser Val Ala Val Asp Val Thr Asn Val Tyr Ile Met Gly Tyr Leu Ala Gly
ACA AAT TAC GCG GAT GAA ACC ATC TCA GTG GCA GTA GAC GTA ACA AAC GTC TAT ATT ATG GGG TAT CTT GCC GGT     343

                            85                                         95
Asp Val Ser Tyr Phe Phe Asn Glu Ala Ser Ala Thr Glu Ala Ala Lys Phe Val Phe Lys Asp Ala Lys Lys Lys
GAT GTG TCC TAT TTT TTC AAC GAG GCT TCA GCA ACA GAA GCT GCA AAA TTC GTA TTC AAA GAT GCT AAG AAA AAA     418

                           110                                        120
Val Thr Leu Pro Tyr Ser Gly Asn Tyr Glu Arg Leu Gln Thr Ala Ala Gly Lys Ile Arg Glu Asn Ile Pro Leu
GTG ACG CTT CCA TAT TCA GGC AAT TAC GAA AGG CTT CAA ACT GCT GCA GGA AAA ATA AGA GAA AAT ATT CCA CTT     493

                           135                                        145
Gly Leu Pro Ala Leu Asp Ser Ala Ile Thr Thr Leu Tyr Tyr Tyr Thr Ala Ser Ser Ala Ala Ser Ala Leu Leu
GGA CTC CCA GCT TTG GAC AGT GCC ATT ACC ACT TTG TAT TAC TAC ACC GCC AGT TCT GCG GCT TCT GCA CTT CTT     568

                           160                                        170
Val Leu Ile Gln Ser Thr Ala Glu Ser Ala Arg Tyr Lys Phe Ile Glu Gln Gln Ile Gly Lys Arg Val Asp Lys
GTA CTC ATT CAA TCC ACG GCT GAA TCT GCA AGG TAT AAA TTT ATT GAA CAA CAA ATT GGA AAG CGT GTA GAC AAA     643

                           185                                        195
Thr Phe Leu Pro Ser Leu Ala Thr Ile Ser Leu Glu Asn Asn Trp Ser Ala Leu Ser Lys Gln Ile Gln Ile Ala
ACT TTT TTA CCA AGT TTA GCA ACT ATT AGT TTG GAA AAT AAT TGG TCT GCT CTG TCC AAG CAA ATT CAG ATA GCC     718

                           210                                        220
Ser Thr Asn Asn Gly Gln Phe Glu Ser Pro Val Val Leu Ile Asp Gly Asn Asn Gln Arg Val Ser Ile Thr Asn
AGT ACC AAT AAT GGA CAA TTT GAG AGT CCT GTT GTG CTT ATA GAT GGT AAC AAC CAA CGA GTC TCT ATA ACC AAT     793

                           235                                        245        ↓
Ala Ser Ala Arg Val Val Thr Ser Asn Ile Ala Leu Leu Leu Asn Arg Asn Asn Ile Ala Ala Ile Gly Glu Asp
GCT AGT GCT CGA GTT GTA ACC TCC AAC ATA GCG TTG CTG CTA AAC AGA AAT AAT ATT GCA GCC ATT GGA GAG GAC     868

                           260
Ile Ser Met Thr Leu Ile Gly Phe Glu His Gly Leu Tyr Gly Ile
ATT TCT ATG ACA CTC ATC GGC TTT GAA CAT GGA CTT TAT GGT ATA TAG TGTAAGTTTAAAGCTATGGACAAGCACAAACTCCA     951


CCTGAAGAACAATCTGTTGTTCTTCGAGAGGTTAATCTACTTGTATAAATAAAGAATGTTCATGTGATCTATCTACGTTAATTCTGTCTGTTGTTGTTG    1050


CTTTAAATAATAAAAAGTGTGGAGTCCTTCTATAAAAAAAAAAA                                                          1094
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FIGURE 1: Nucleotide and amino acid sequence encoding BD1 cDNA. The BD1 nucleotide sequence codes for a 23 amino acid signal peptide followed by a protein of 267 residues. Nucleotides 802−804 represent the stop codon and 805−982 are 3′ noncoding sequences. The two underlined AATAAA hexanucleotide sequences in the 3′ noncoding region correspond to polyadenylation signals found in two separate clones. The arrow indicates the putative cleavage site between amino acids 247 and 248.

utilized different consensus polyadenylation signals (Figure 1). The cDNA clones obtained from 5′ RACE extended 57 bp upstream of the sequences encoding the mature amino terminus of the BD1 protein. The BD1 cDNA encoded a signal peptide (amino acids −23 to −1) followed by sequences encoding 267 amino acids ending in a stop codon.

Sequence Homology between RIPs. The deduced protein sequence of BD1 was found to be homologous to many RIPs including trichosanthin, momorcharin, luffin a, gelonin, and ricin A, which have 85%, 68%, 64%, 34%, and 33% amino acid identity with BD1, respectively (Figure 2). Not unexpectedly, the other *Curcubitaceae* family members trichosanthin (Collins et al., 1990; Shaw et al., 1991), momorcharin (Ho et al., 1991), and luffin a (Kataoka et al., 1992) have the highest sequence identity to BD1. There are five main regions of high sequence alignment in these RIPs, matching amino acids 4−25, 62−74, 109−128, 159−168, and 189−193 of BD1. The last four regions cluster around the putative active site cleft found in ricin A and trichosanthin (Shaw et al., 1994; Wong et al., 1994). Unlike all the other RIPs listed in Figure 2 which have an alanine at residue 161, BD1 contains a serine residue at this position.

Expression and Analysis of rBD1. Recombinant $BD1^{267}$ (expressed by plasmid pSE 14.0 and encoding residues 1−267) and $rBD1^{247}$ (contained in plasmid pSE 13.0 and encoding residues 1−247) were constructed (Figure 3) and expressed in *E. coli* as described in Experimental Procedures. Preliminary localization experiments showed that >90% of the rBD1 protein localized to the insoluble cell fraction, necessitating denaturation and refolding to generate biologi-

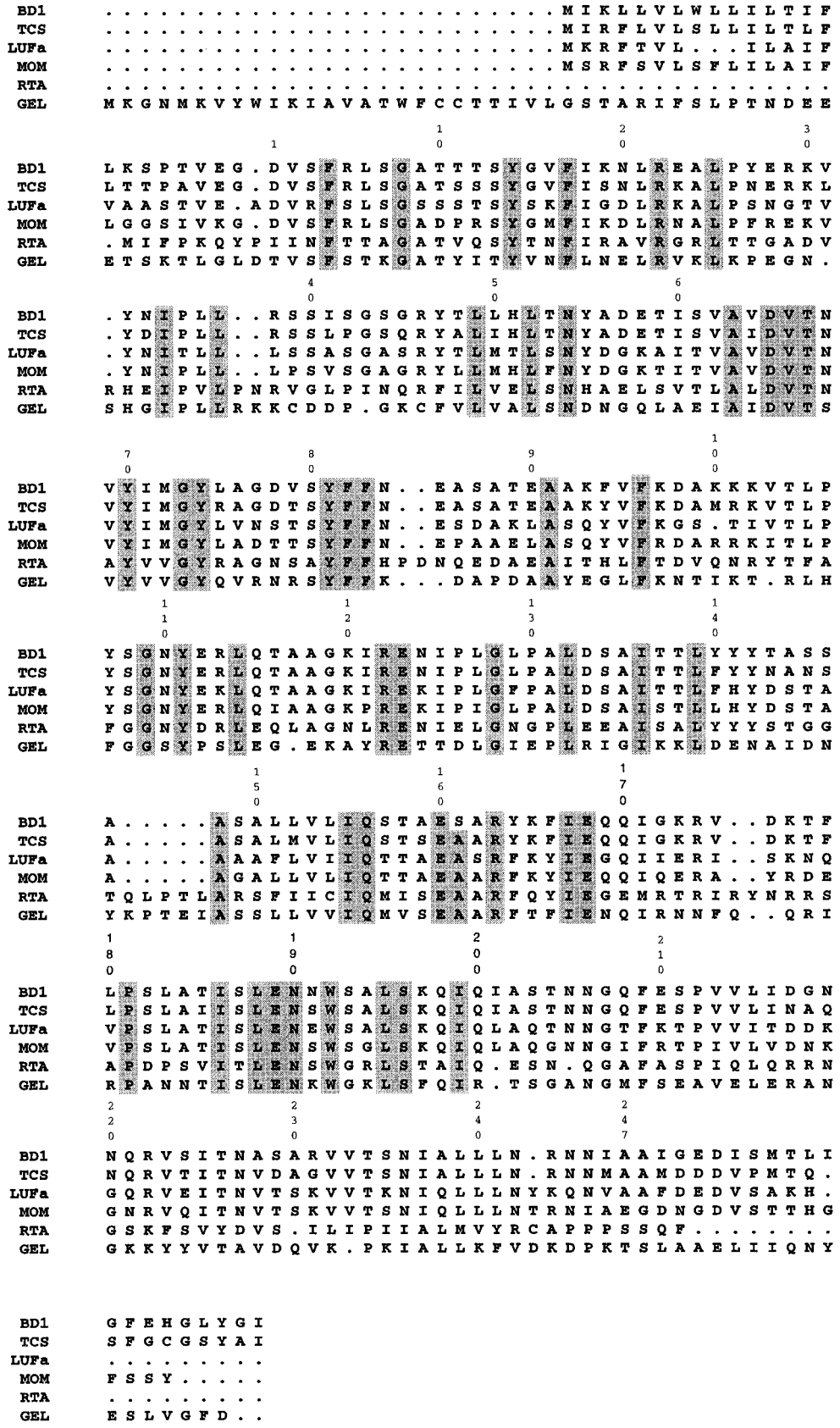

FIGURE 2: Amino acid sequence alignment of various RIPs. Sequences were aligned to show maximum homology with invariant residues shaded. Trichosanthin (TCS), luffin a (LUFa), and momorcharin (MOM), including a 23 amino acid signal sequence, are aligned with BD1 starting from their mature amino termini. RTA = ricin A chain; GEL = gelonin.

cally active rBD1. A single CM-Sepharose chromatography step purified the proteins to near homogeneity as seen by 12% SDS−PAGE under nonreducing conditions, which revealed a single protein band migrating at approximately 29 kDa for $rBD1^{267}$ and 27 kDa for $rBD1^{247}$ (Figure 4). The elution conditions for the two forms differed slightly with $rBD1^{267}$ eluting at 0.15 M NaCl and $rBD1^{247}$ eluting at 0.2 M NaCl. The rBD1 forms stained positive in immunoblot

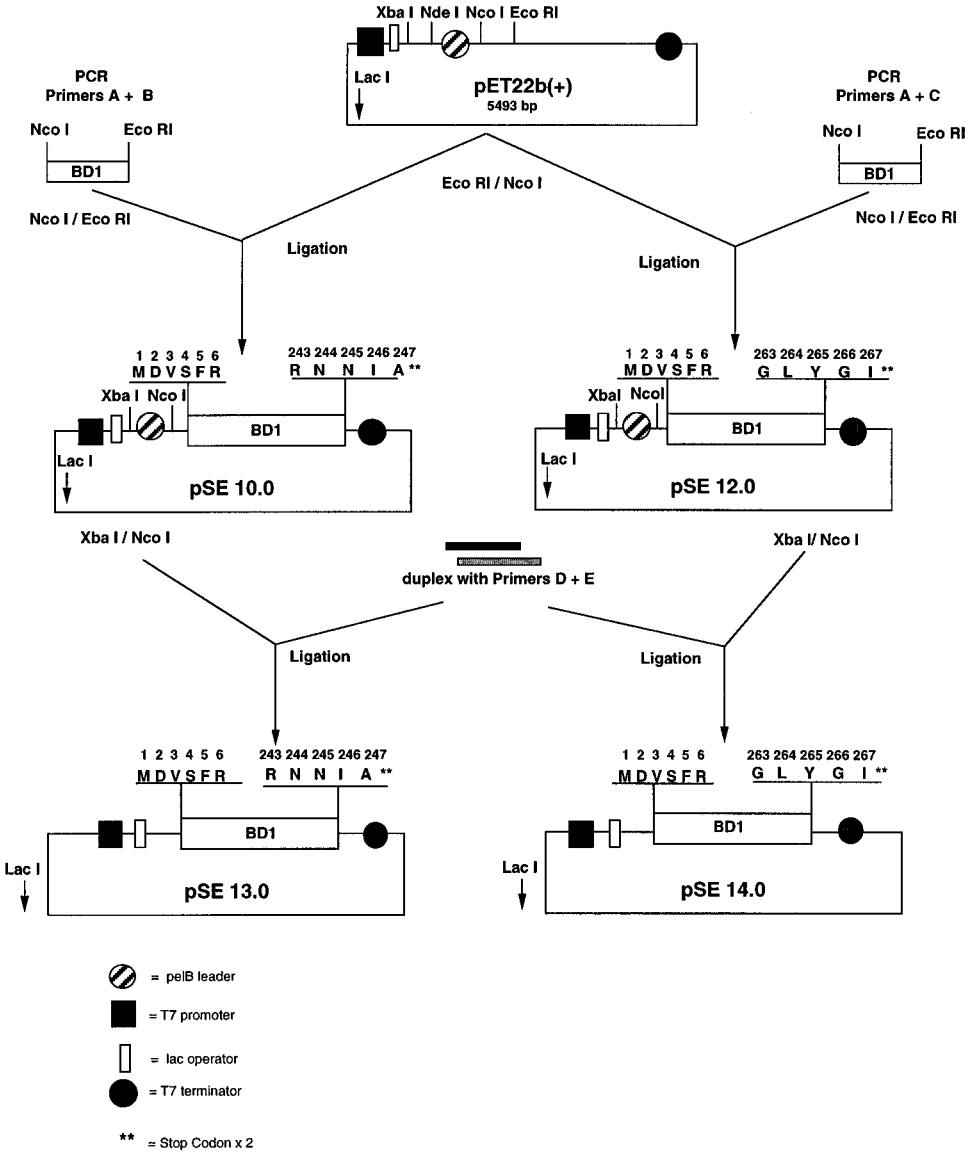

FIGURE 3: Construction of rBD1 expression plasmids. Plasmids pSE 10.0 and pSE 12.0 contain BD1 sequences from amino acids 1−247 and 1−267, respectively, plus a pelB leader sequence, and are under the control of T7 promoter and lac operator. Plasmids pSE 13.0 and pSE 14.0 contain BD1 sequences from amino acids 1−247 and 1−267, respectively, but without the pelB leader sequence. Primer A = 5′-GTCAGAGTTCCATGGATGTGAGCTTTCGTTTATCAGGTGCTACAACCACATCCTAT-3′; primer B = 5′-CAAAGATCCTCT-GAATTCTTATTATGCAATATTATTTCTGTTAGCAGCAA-3′; primer C = 5′-CAAAGATCCTCTGAATTCTTACTATATAC-CATAAAGTCCATGTTCAAAGCCGATGAGTGTCATAGAAATGTCCTC-3′; primer D = 5′-CTAGAAATAATTTTGTTTAACTTTAA-GAAGGAGATAC-3′; primer E = 5′-CATGGTATCTCCTTCTTAAAGTTAAACAAAATTATTT-3′.

analysis using polyclonal rabbit anti-BD antiserum generated from native BD1 as immunogen. Yields of purified rBD1 ranged from 2 mg/L of shake flask culture to 25 mg/L culture by fermentation.

Both $rBD1^{267}$ and $rBD1^{247}$ inhibited protein synthesis in a cell-free rabbit reticulocyte lysate system with EC_{50} values of approximately 10 pM, which is nearly identical to that of native BD1 protein (Table 1). Trophoblasts and choriocarcinoma cells have been shown to be sensitive to the cytotoxic activity of RIPs (Battelli et al., 1992). JAR choriocarcinoma cells were sensitive to native BD1 as determined by protein synthesis inhibition with an EC_{50} value of 0.17−0.34 μM. Recombinant $BD1^{267}$ and $rBD1^{247}$ were similarly active at inhibiting protein synthesis in JAR cells.

Activity of rBD1 Mutant. Based on the extensive homology of BD1 to other RIPs, glutamate 189 was predicted to be involved in the *N*-glycosidase activity. A mutant was constructed from $rBD1^{247}$ in which the residue was changed to lysine ($rBD1\ Lys^{189}$). The mutant protein was expressed in *E. coli* and purified as described for $rBD1^{247}$ (data not shown). The single amino acid substitution at residue 189 resulted in a 10-fold decrease in catalytic activity in comparison with $rBD1^{247}$, as determined by protein synthesis inhibition assays (Table 1). $rBD1\ Lys^{189}$ was unable to inhibit cellular protein synthesis in JAR cells at up to 1.36 μM.

Rodent Toxicity. BD1 purified from the roots of *B. dioica* was previously reported nontoxic to mice when administered intravenously up to 40 mg/kg (Siegall et al., 1994a). To determine whether rBD1 was also similarly nontoxic to mice, toxicology studies were performed. The toxicity of rBD1 was also evaluated in rats, which have been a useful model to study the dose-limiting toxicity of PE40-based immunotoxins, namely, vascular leak syndrome (Siegall et al., 1994b). The LD_{50} value of rBD1 in mice was approximately 40 mg/kg and >25 mg/kg in rats (Table 2). Larger scale

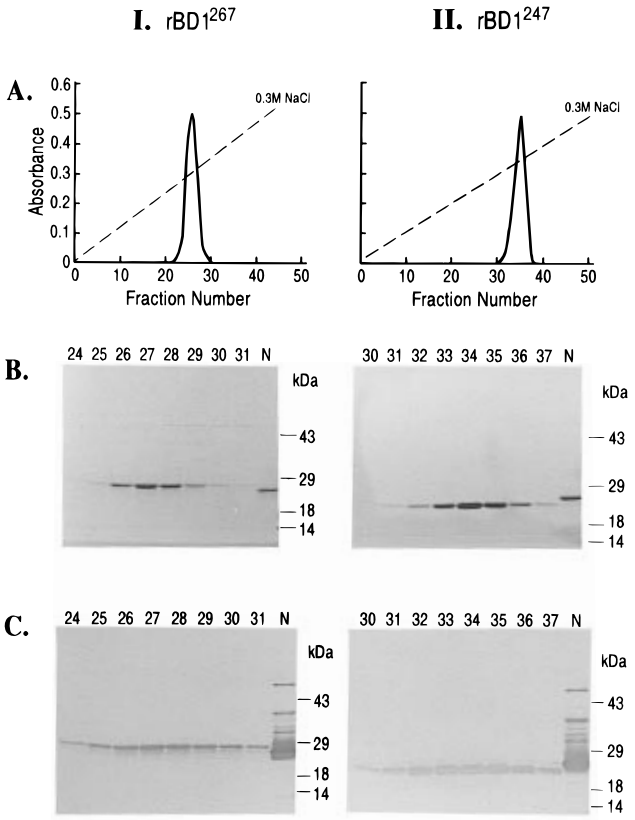

FIGURE 4: Purification of rBD1. (A) CM-Sepharose chromatography (0−0.3 M NaCl gradient). Dashed lines correspond to a NaCl gradient. Absorbance at 280 nm was determined. (B) 12% SDS−PAGE (nonreduced) stained with Coomassie brilliant blue. (C) Immunoblot analysis stained with rabbit anti-BD1 polyclonal antisera. N = native BD1.

Table 1: Inhibition of Protein Synthesis by BD1[a]

RIP	EC_{50} RRL (cell-free)	EC_{50} JAR (cell-based)
native BD1	7−10 pM	0.17−0.34 μM
$rBD1^{267}$	8−12 pM	0.17−0.34 μM
$rBD1^{247}$	7−10 pM	0.17−0.34 μM
$rBD1^{247}Lys^{189}$	80−120 pM	>1.36 μM

[a] The EC_{50} value represents the amount of BD1 required to inhibit 50% of protein synthesis in either RRL (rabbit reticulocyte lysate) or JAR (choriocarcinoma) assays. Data were determined by [^{3}H]leucine incorporation into translation products (RRL) or cellular protein (JAR) relative to untreated samples. The EC_{50} results represent the range of two or three experiments done in duplicate or triplicate.

Table 2: Rodent Toxicity[a]

toxin	LD_{50} (mg/kg) mice	rats
rBD1	40	>25
PE40	2.5	2.0[b]
dgRA	5[c]	<5

[a] The LD_{50} value represents the amount of protein toxin that results in lethality in 50% of injected animals. Animals were observed for >7 days following administration, and for each LD_{50} value, 8−20 animals were used. dgRA = deglycosylated ricin A chain. [b] Data from Siegall et al. (1997). [c] Data from Siegall et al. (1994a).

production of rBD1 resulting in material of concentrations >5 mg/mL will be necessary to facilitate injection of larger doses into rats. The toxicity of deglycosylated ricin A chain and PE40 was also evaluated in mice and rats for comparison purposes. The LD_{50} of dgRA in mice was reported to be 5 mg/kg (Siegall et al., 1994a). Rats were more sensitive to dgRA than were mice with survival observed in 1/5 rats injected at 5 mg/kg (and 0/5 rats at 10 mg/kg); thus the LD_{50} is <5 mg/kg (Table 2). The LD_{50} of PE40 in mice was found to be 2.5 mg/kg, in agreement with previously reported data (Hwang et al., 1987), and in rats, the LD_{50} value was 2.0 mg/kg.[4]

Table 3: Comparison of RMS Differences (Å) between Cα Positions for RIPs[a]

	rBD1 mol A	rBD1 mol B	trichosanthin
rBD1 mol B	0.3		
trichosanthin	0.4	0.4	
ricin A chain	1.2	1.2	0.9

[a] Calculated with the program ALIGN (Satow et al., 1986). Coordinates for trichosanthin are from PDB entry 1MRJ[3]; those for ricin A chain are from PDB entry 1RTC (Mlsna et al., 1993).

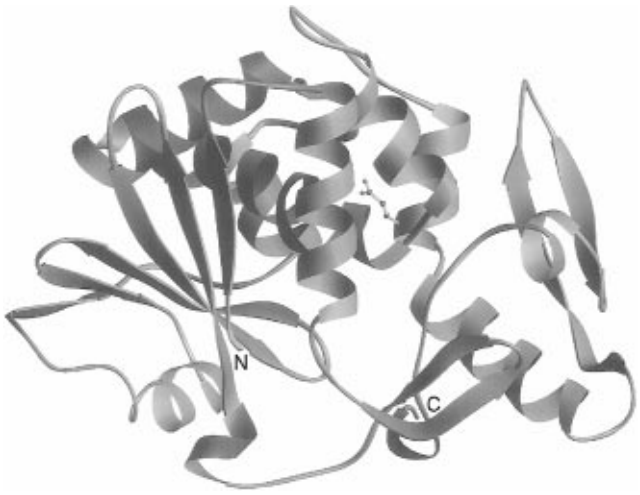

FIGURE 5: Molecular model of $rBD1^{247}$. A RIBBONS (Carson, 1991) representation of the tertiary structure of rBD1 for one of the two molecules in the asymmetric unit. The N- and C-termini are labeled, and the side chain of glutamate 189 is shown. The RIBBONS color scheme corresponds to specific secondary structural elements as follows: cyan, α helix; green, β strand; blue, 3_{10} helix; orange, random coil or loop or turn.

Structural Analysis of $rBD1^{247}$. The rBD1 molecule is structurally homologous to other type I RIPs and to the A chains of type II RIPs. Comparisons of the $rBD1^{247}$ structure with the structures of trichosanthin[3] and ricin A chain (Mlsna et al., 1993) show the same levels of similarity found between other RIP structures as shown in Table 3. The RMS difference between Cα positions for rBD1 molecules A and B is essentially the same as the differences between either rBD1 molecule and the fellow type I RIP trichosanthin. The deviations of both rBD1 molecules and trichosanthin (type I RIPs) from ricin A chain (type II RIP) are more significant, but comparable. A RIBBONS diagram (Carson, 1991) of the $rBD1^{247}$ structure is shown in Figure 5.

The N- and C-termini of rBD1 are in different intramolecular environments. The N-terminus is only partially exposed at the surface of the molecule, and the N-terminal residues form the outer strand of a six-stranded β sheet that is an integral part of the structure. The C-terminus does not appear to play as critical role in the protein structure but rather protrudes from the molecule, executing a single helical turn in the process. Analysis of the temperature factors at this stage suggests that both the N- and C-terminal residues

are mobile; however, the temperature factors away from the N-terminus quickly settle to values seen in other well-anchored sections of the structure. The crystal structure of rBD1 without substrate does not provide an explanation of the loss of activity associated with the rBD1 Lys^{189} mutation. Because of steric and charge considerations, it seems unlikely that the side chain of lysine 189 would adopt a conformation similar to that found for glutamate 189 in this structure.

DISCUSSION

Bryodin 1 is a type I RIP that, due to its extremely low animal toxicity and potency in inhibiting protein synthesis, represents a component of immunotoxins that has potential advantages over other commonly used plant RIPs and bacterial toxins (Siegall et al., 1994a). In this report, a complete cDNA sequence encoding BD1 was obtained from *B. dioica* mRNA using a cloning procedure that involved three PCR steps. First, a small region of exact BD1 sequence was generated by PCR amplification using degenerate oligonucleotides designed from the N-terminal protein sequence. Using this information to generate exact oligonucleotides, 3′ and 5′ RACE yielded overlapping DNA fragments that encompassed the entire BD1 cDNA sequence which encodes a 267 amino acid protein and a 23-residue signal peptide on the amino terminus (Figure 1).

BD1 was found to be homologous to many type I RIPs, most notably to the other *Cucurbitaceae* family members trichosanthin, luffin a, and momorcharin (Figure 2). Trichosanthin was especially related with 85% identity between residues 1 and 267 of BD1 and residues 1 and 266 of trichosanthin. Like BD1, the complete cDNA sequence of trichosanthin shows the presence of a 23 amino acid signal peptide (Chow et al., 1990). Trichosanthin is processed between residues 247 and 248 to generate a mature 247 amino acid protein (Collins et al., 1990). Based on the near identity of residues 230−247 with trichosanthin, BD1 may also be processed to a 247 amino acid mature form. Isolated from *B. dioica* roots, mature BD1 has a molecular mass of 28.2 kDa (±2.7 Da) as determined by electrospray mass spectrometry (data not shown). This indicates that even discounting the additional mass due to glycosylation in native BD1, a 267-residue mature form is unlikely, thus indicating that BD1 is most likely processed proteolytically.

To evaluate BD1 as a recombinant protein, two molecular forms composed of amino acids 1−267 or 1−247 were produced in *E. coli*. Interestingly, $rBD1^{267}$ eluted from CM-Sepharose with lower NaCl concentrations (0.14−0.16 M NaCl) than did $rBD1^{247}$, which eluted at 0.19−0.20 M NaCl (Figure 4A). This same trend was observed for trichosanthin when recombinant forms composed of residues 1−247 (form I) and 1−266 (form II) were compared (Zhu et al., 1992). However, in contrast to $rBD1^{267}$ which required NaCl for elution, the trichosanthin form II lost most of its affinity for CM-Sepharose and eluted in the column washing step prior to NaCl addition. Both rBD1 forms had identical protein synthesis inhibitory activity in comparison with native BD1 protein (Table 1). Thus, amino acids 248−267 do not contribute significantly to the catalytic activity of rBD1. This contrasts with trichosanthin, in which native protein and a mature recombinant protein of 247 residues were 5-fold more active than when expressed as a 266 amino acid recombinant protein (Zhu et al., 1992). Additionally, the ability to inhibit cellular protein in JAR cells was not affected by the removal of the 20 residues on the C-terminus of BD1 (Table 1).

The alignment of BD1 with other RIPs has lent insight into possible structure−function relationships, especially in the active site of the protein. Glutamate at residue 189 is conserved in most RIPs, both type I and type II. Mutations of glutamate at position 189 in trichosanthin in which aspartate or alanine was substituted in place of glutamate resulted in a marginal reduction (12%) in catalytic activity and therefore was suggested not to be directly involved in catalysis (Wong et al., 1994). Similar findings were observed for glutamate at residue 208 (aligned with glutamate 189 in BD1 and trichosanthin) of ricin A chain in which the reduction in activity following mutation to aspartate was minimal, indicating that the catalytic importance of this residue was conserved but not invariant (Frankel et al., 1990). In contrast to trichosanthin and ricin A chain, substitution of lysine at residue 189 in BD1 resulted in a 10-fold decrease in catalytic activity, indicating its involvement in catalytic activity (Table 2).

RIPs including bryodin, trichosanthin, pokeweed antiviral protein, saporin, and gelonin are toxic to choriocarcinoma cells and primary trophoblasts, as well as having abortifacient activity (Battelli et al., 1992). The $rBD1^{247}$ and $rBD1^{267}$ forms retained similar activity against JAR choriocarcinoma cells in comparison with native BD1 (Table 1). The rBD1 Lys^{189} mutant was not able to inhibit protein synthesis up to 1.36 μM, indicating that the cytotoxicity of BD1 toward JAR cells requires its protein synthesis inhibition activity.

Bryodin has been reported to inhibit the growth of HIV-infected cells and to reduce HIV production (Wachinger et al., 1993). Other RIPs including trichosanthin (McGrath et al., 1989) and GAP31 from *Gelonium multiflorum* (Lee-Huang et al., 1994) also exhibit anti-HIV properties. Trichosanthin has been clinically tested for anti-HIV activity (Kahn et al., 1994). There was an indication of anti-HIV activity in patients receiving doses of 36 and 50 μg/kg, which enabled trichosanthin to reach a target concentration in serum that can result in antiviral activity. Despite the extensive sequence homology between BD1 and trichosanthin, it is unknown whether rBD1 would have similar clinical activity.

Vascular leak syndrome (VLS) is the dose-limiting toxicity associated with many different immunotoxin clinical trials (Siegall et al., 1994b). We were especially interested in evaluating the toxicity of rBD1 in rats since VLS was induced by BR96 sFv−PE40 or PE40 itself[4] upon administration in rats. No toxicity or any evidence of VLS was apparent following administration of up to 25 mg/kg rBD1 (Table 2). Ricin A chain was toxic to rats with an LD_{50} of $<$5 mg/kg, although there was scant evidence of pulmonary hydrothorax fluid that was evident upon administration of PE40 at ∼2 mg/kg. Thus, while rats may be predictive of VLS for PE40-based immunotoxins, they are not predictive of the ability of RIP-based immunotoxins to induce VLS. Nonetheless, the difference in rat toxicity between rBD1 and both ricin A chain and PE40 is striking and may ultimately enable BD1-based immunotoxins to be constructed which have minimal side effects and enhanced therapeutic windows.

Single-chain immunotoxin fusion proteins utilizing rBD1 are being constructed and will be tested *in vitro* and *in vivo* for both efficacy and toxicity. Analysis of the rBD1 crystal structure suggests that fusion to the N-terminus is likely to imperil the stability of the tertiary structure, facilitating a

disruption of the N-terminal strand that could spread to the surrounding parts of the structure, unless a sufficiently long and innocuous peptide extension is provided. A fusion to the C-terminus does not appear to require so long an extension or to so threaten the rBD1 structure.

Expression and characterization of saporin (Barthelemy et al., 1993) and gelonin (Better et al., 1994), and subsequently fusion toxins utilizing these RIPs, have been reported (Lappi et al., 1994; Better et al., 1995). A single-chain fusion toxin composed of basic fibroblast growth factor and saporin was found to be specifically active in killing target cells (Lappi et al., 1994). Fusion toxins composed of the gelonin and a single-chain antibody derived from H65, a murine anti-CD5 antibody, were reported to be cytotoxic in both sFv-gelonin and gelonin-sFv formats against certain T-cell lines and peripheral blood mononuclear cells (Better et al., 1995). Since BD1 has an excellent toxicity profile *in vivo* as well as possessing potent protein synthesis inhibition activity once inside a cell, its use in immunotoxins may provide for a wider therapeutic window than other RIPs.

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