Engineering of the RTB Lectin as a Carrier Platform for Proteins and Antigens

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Plant Physiology, Pathology, and Weed Science

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January 26, 2007
Blacksburg, VA

Keywords:
Ricin, ricin B-chain, RTB, lectin, drug carrier, antigen carrier, Agrobacterium-mediated transient expression, plant-based bioproduction, adjuvant, lectin-mediated uptake, retrograde trafficking, Type II RIP processing, capture/carry platform, N. benthamiana, Immunoglobulin scaffolding
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Abstract

The major obstacle many promising drugs struggle to overcome is the barrier imposed by the outer cell membrane. In addition to technologies such as liposomes and cell-penetrating peptides, more attention is being given to the class of proteins known as lectins to deliver therapeutic and antigenic proteins to the interiors of cells. Lectins bind to but do not modify sugars, and provide an efficient route to endocytosis. The galactose/N-acetyl-galactosamine specific lectin ricin B-chain (RTB) is especially attractive in possibly fulfilling a carrier role due to its well-characterized endocytotic trafficking and its efficacy over a wide range of cell types. By producing RTB recombinantly in plants it is possible to create a fully active, non-toxic carrier that does not rely on the processing of large amounts of toxic material (e.g. castor bean). Payload molecules such as small molecules and proteins can be attached to RTB via chemical conjugation at primary amine groups, without the loss of lectin or uptake activities. The biotin/streptavidin interaction and direct genetic fusion of polypeptides also provide efficient mechanisms for the attachment of payload proteins to RTB. An immunoglobulin domain-based scaffolding mechanism bridges modified RTB and payload proteins when co-expressed in Agrobacterium-infiltrated plant leaves. Carrier and payload proteins expressed in plants and E. coli, respectively, and purified independently are not able to assemble into an efficient carrier/payload arrangement. These findings show that plant cells are able to correctly produce the two components of the carrier/payload system and assemble them into an efficient and flexible capture and carry technology.
Acknowledgements

First and foremost, I would like to thank Dr. Carole Cramer for the opportunity of working in her lab. Her style, work ethic, and reasoning ability has been an inspiration and effective motivator for me. She always allowed me to follow my own ideas and never settled for inside-the-box thinking. All of this combined for an atmosphere in which I was motivated to produce timely good science, but was never micro-managed.

I would like to thank my committee members for all of their invaluable input. I would especially like to thank Dr. Nessler for his dealing with all of the forms that I could not always take care of, because I lived 700 miles from campus.

I thank my family (Mom, Dad, and Karen) for, quite simply, being the most wonderful people on the face of this (or any other) planet.

My deepest and most heartfelt thanks to Shannon Hill, for keeping the plants alive and more importantly, for providing the greatest support and understanding I could ever hope for, and especially for putting your life on hold to come with me to Arkansas for two and half years. You’re the best. I cannot express here how much you mean to me.

I would like to extend special thanks to Dr. Jianyun Liu, for always meaningful, but maybe not always civilized, conversations about US/China relations, American culture (or lack thereof), George W. Bush, and experimental strategies. I would like to acknowledge and thank Jorge Ayala for the work he has done, and continues to do, in regards to the RTB breakdown research. I wish you both the best.

My thanks go out to all of the members of the Cramer Lab, past and present, especially Selester Bennett for enlightening and lively discussions on topics ranging from protein purification to politics. Many thanks to Niki McMaster and Jenny Jennrette from the VT Cramer Lab, and Dr. Maureen Dolan, Melissa Russel, Ross Fergus, and Dr. Giuliana Medrano from the AR Cramer Lab.
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List of Abbreviations

32BP: 32 kilodalton breakdown product
aa: amino acid
AdIII: C-terminal domain of ricin A-chain
bME: beta-mercaptoethanol
CaMV: cauliflower mosaic virus
cbRTB: castor bean-derived ricin B-chain
C\textsubscript{H}1: first constant domain of immunoglobulin heavy chain
C\textsubscript{L}: constant domain of immunoglobulin light chain
CPP: cell penetrating peptide
CT: cholera toxin
CTL: cytotoxic T-lymphocyte
DTT: dithiothreitol
ER: endoplasmic reticulum
ERAD: endoplasmic reticulum-associated degradation
ERT: enzyme replacement therapy
Fab: portion of immunoglobulin that binds antigen
Fd: region of immunoglobulin heavy chain composed of the variable and first constant domains
FITC: fluorescein isothiocyanate
FPLC: fast protein liquid chromatography
GFP: green fluorescent protein
Glut\textsubscript{red}: reduced glutathione
GST: glutathione-S-transferase
HBSS: Hank’s Buffered Salt Solution
HC: immunoglobulin heavy chain
HRP: horseradish peroxidase
IFN-g: interferon-gamma
Ig: immunoglobulin
IL-10: interleukin 10
IL-12: interleukin 12
List of Abbreviations, continued

IPTG: Isopropyl β-D-1-thiogalactopyranoside
kD: kilodalton
κLC: mouse kappa light chain
LC: immunoglobulin light chain
LEA: lectin from tomato
MHC: major histocompatibility complex
MLI: mistletoe lectin I
NHS: N-hydroxysuccinimide
OVA: ovalbumin
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
RCA\textsubscript{60}: *Ricinus communis* toxin (ricin)
RIP: ribosome inactivating protein
RTA: ricin A-chain
RTB: ricin B-chain
rRTB: recombinant ricin B-chain
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sp: patatin signal peptide
SPD: storage protein domain
T-DNA: transferred DNA (by *Agrobacterium*)
TEV: tobacco etch virus translational enhancer
TSP: total soluble protein
UEA-1: *Ulex europaeus* lectin 1
WGA: wheat germ agglutinin
I. Lectins as carrier molecules

The major limitation many promising drugs struggle to overcome is the barrier of the cell membrane. In order for many drugs to work, they must first localize to the area where its bioactivity is required. An enzyme that performs a particular required function in the cytosol can not be effective if it never gets to the cytosol. Diverse approaches to delivering drugs and antigens to the interior of the cell exist, including liposomes, cell-penetrating peptides (CPPs) and lectins. Liposomes rely on the chemical properties of membranes and membrane fusion mechanisms to gain access to the cytosol. Cell penetrating peptides (CPPs) comprise a chemically distinct technology. CPPs are short (< 30 residues) peptides containing elevated amounts of the basic amino acids lysine and arginine. The mechanism of uptake is “energy independent”, that is, CPP uptake is not affected by inhibitors of the endocytosis pathway. CPPs composed of D-amino acids exhibited equal or greater rates of uptake than those composed of “normal” L-amino acids, indicating that the uptake is not receptor-mediated.\(^1\) More recent studies suggest that the mode of internalization is dependent upon the “cargo” being delivered. Larger cargoes such as whole proteins appeared to enter through an endosomal route, while peptide cargoes diffused through the membranes in a manner dependent upon membrane potential.\(^2\) One advantage of these systems over a protein-based system is that it is unnecessary to employ a complex and expensive protein expression system, as liposomes and CPPs can be synthesized \textit{de novo}. A major drawback of these systems is the requirement for hydrophobic or small cargoes as in liposomes, and an unpredictable mode of internalization in CPPs.
Lectins, a class of proteins which bind to but do not modify sugars, are attractive as the basis for a protein-based delivery system. Plants serve as a rich source for finding useful lectins, but lectins are found in all types of cells, eukaryotic as well as prokaryotic. Many different types of sugars extensively decorate the outer cell surface, in the form of glycans on outer membrane proteins or glycosylated membrane phospholipids. Binding to these sugars by lectins in some cases induces endocytosis, thereby bringing the lectin and whatever may be bound to it inside the cell. The variation in lectin specificity is quite large, and new lectins with different specificities are constantly being discovered and characterized. Different lectins also exhibit different internalization mechanisms, so the choice of lectin is dependent upon the route one wishes to deliver the drug. Table 1 lists a variety of lectins and their uses, as described in Bies, et al. (2004)

<table>
<thead>
<tr>
<th>Source (abbrev.)</th>
<th>Specificitya</th>
<th>Target area</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ricinus communis</em> (RCA60)</td>
<td>D-gal, GalNac</td>
<td>Mucosa</td>
</tr>
<tr>
<td><em>Viscum album</em> (MLI)</td>
<td>D-gal, GalNac</td>
<td>Mucosa</td>
</tr>
<tr>
<td><em>Triticum vulgaris</em> (WGA)</td>
<td>GlcNac, NeuNAc</td>
<td>GIb</td>
</tr>
<tr>
<td><em>Canavalia ensiformis</em> (ConA)</td>
<td>D-Man, D-Glc</td>
<td>GI</td>
</tr>
<tr>
<td><em>Lycopersicum esculentum</em> (LEA)</td>
<td>GlcNac</td>
<td>mucosa</td>
</tr>
<tr>
<td><em>E. coli</em> (LTB)</td>
<td>D-gal</td>
<td>GI</td>
</tr>
<tr>
<td>Cholera toxin (CT)</td>
<td>GM1 ganglioside</td>
<td>GI</td>
</tr>
</tbody>
</table>

a D-gal: galactose; GalNac: N-acetyl-galactosamine; GlcNac: N-acetyl-glucosamine; NeuNAc: N-acetyl-sialic acid; D-man: mannose; D-glc: glucose  
b GI: gastro-intestinal tract

An interesting area of research currently is the use of lectins conjugated to nanoparticles. Yin, et al. have successfully conjugated wheat germ agglutinin (WGA), a N-acetyl-glucosamine- and sialic acid-specific lectin from *Triticum vulgare*, to nanoparticles through carbodiimide chemistry. The nanoparticles were then loaded with thymopentin, a pentapeptide used in treatment of some autoimmune disorders. Yin et al. found that immuno-suppressed rats treated with lectin-coated nanoparticles showed an elevated exposure to thymopentin compared to non-
coated nanoparticles or thymopentin alone. This suggests that lectins can enhance the efficacy of nanoparticle delivery.\textsuperscript{5}

We are interested in exploiting the cell uptake and trafficking properties of the plant lectin ricin B-chain, as a possible antigen and therapeutic protein delivery platform.

II. Ricin toxin

Ricin is a heterodimeric protein toxin found in the seeds of the castor bean plant \textit{Ricinus communis}. Ricin toxin belongs to, and is the model for, the type II ribosome inactivating proteins (type II RIPS). Type II RIPS are characterized by a heterodimeric structure comprised of an enzyme toxin and a lectin which mediates uptake of the toxin by target cells. The 32 kD A-chain (RTA) is an N-glycosidase that preferentially de-purinates a specific residue, A\textsuperscript{4324} of the 28 S ribosomal subunit (in rat), which halts protein synthesis by destroying an elongation factor binding site.\textsuperscript{6} The 32 kD B-chain (RTB) is a galactose/N-acetyl-galactosamine-specific lectin.\textsuperscript{7} Both RTA and RTB are glycosylated (RTA contains one glycan at Asn-10; RTB contains two glycans at Asn-95 and Asn-135). All of these glycans are terminated in mannose\textsuperscript{8}, which has been shown to be a possible alternative route to endocytosis through the cell-surface D-mannose receptor.\textsuperscript{9} RTA without its partner RTB is not toxic to whole cells, as RTB is required for delivery of RTA to the cytosol of target cells, where it can access its substrate ribosomes. RTB alone is likewise not toxic, as it contains no activity other than binding to galactose/N-acetyl-galactosamine, which in itself is not toxic.\textsuperscript{10} However ricin holotoxin is extremely toxic, as the two subunits working together produce an effective protein synthesis-halting mechanism. Figure I shows the crystal structure of ricin and two other type II RIPS, mistletoe lectin I and ebulin. The position of the glycans and galactose binding sites on RTB are shown, as well as the position
of the interchain disulfide bond. Note the similarity in structure between ricin, mistletoe lectin I and ebulin.

Ricin, like most other type II RIPs, is encoded on a single gene containing no introns. The initial gene product, preproricin, is a single polypeptide composed of a signal peptide, RTA, a short linker, and RTB. Following insertion of the nascent preproricin into the ER lumen during translation, the signal peptide is cleaved to give proricin. Inside the ER lumen, proricin is glycosylated once on the RTA moiety and twice on the RTB moiety, and RTA and RTB are joined by a single disulfide bond. Proricin contains a 12 amino acid (aa) linker peptide between RTA and RTB that contains vacuolar sorting information. Once in the vacuole, this linker peptide is cut out to yield activated ricin holotoxin. RTA and RTB are joined via the single disulfide bond. Note the similarity in structure between ricin, mistletoe lectin I and ebulin.

Figure 1. The type II Ribosome Inactivating Proteins (RIPs). The picture to the left shows ricin, RTA is colored red and RTB blue. The location of the disulfide bond, the glycans on RTB, and the galactose binding sites are shown. The two pictures on the right are the type II RIPs mistletoe lectin I (top) and ebulin (bottom). Note the similarities in all three structures. Images created in VMD using the following Protein Data Bank files: 2AAI (ricin), 2MLL (mistletoe lectin I), and 1HWO (ebulin).
disulfide bond and through numerous hydrophobic and polar interactions occurring at the RTA – RTB interface. \(^{14}\) Studies by Sehnke, et al. have shown that tobacco plants transformed with the preproricin gene can efficiently process preproricin to active ricin holotoxin, indicating that transgenic tobacco is an effective system for production of recombinant ricin. \(^{15}\) Figure 2 is a representation of ricin toxin processing in castor bean seeds.

Ricin also undergoes complex trafficking and processing following exposure to a target organism. Toxicity is dependent upon retrograde transport through the Golgi to the endoplasmic reticulum (ER) of infected cells. Upon exposure to ricin by the target organism, whether by ingestion, inhalation, or transdermal penetration, the RTB portion of ricin toxin binds to cell-
surface galactose/galactosamine residues. This binding triggers endocytosis of ricin toxin into the cell. Once internalized, ricin enters three separate pathways (see Figure 3). A large portion of the ricin cycles between lysosomes, endosomal compartments, and the cell surface. A portion of the late-endosomal population is transported to lysosomes and is likely degraded. The toxic effects of ricin arise from the movement of the toxin into the retrograde pathway by locating to the Golgi apparatus. Much research has been done studying the toxic effects of ricin in mammalian cell systems. Once in the Golgi, RTB binds to calreticulin and is transported to the ER in a “piggy-back” fashion dependent upon the KDEL sequence of calreticulin. Once in the ER, RTA and RTB are separated via reduction of the disulfide bond by protein disulfide isomerase. RTB is thought to be degraded at this point. Independent RTA translocates from the ER lumen to the cytosol via the Sec61p complex. Point mutation studies have shown that Pro-250 of RTA plays a key role in this translocation, and furthermore the low lysine content of RTA allows it to escape the ER-associated degradation pathway (ERAD) to gain access to the cytosol. A third possible route for endocytosed ricin is the transcytosis pathway, in which ricin travels through one cell to interact with another. This particular pathway is important in epithelial layers. Unpublished data from our group demonstrates that RTB fused to mouse interleukin 12 (IL-12) can pass through HT-29 epithelial cells (Liu & Cramer, 2006). Figure 3 is a cartoon of the three possible routes of internalized ricin and their potential importance in vaccine applications. In antigen presenting cells (APC), which are immune cells that process foreign proteins and molecules and stimulate response to them by presentation of fragments on the surface to T- and B-cells, the retrograde pathway leads to major histocompatibility complex (MHC) class I presentation, and the lysosomal pathway leads to MHC class II presentation.
Targeting of these routes is important to researchers who are interested in utilizing ricin and RTB as an antigen delivery system in possible vaccine applications.

Nature has devised a clever way to gain access to the interior of cells in RTB. It should be possible to exploit the characteristics of cellular uptake of RTB while simultaneously eliminating the potent toxicity of RTA. It is the aim of our research to develop an effective delivery system based on RTB. By utilizing recombinant technology, it is possible to produce an RTB carrier system without ever coming into contact with RTA of any form. This system would be effective and safe, with applications ranging from delivery of therapeutic drugs to antigen delivery in vaccine formulations.
III. Lectins as adjuvants

An adjuvant is any substance that increases the immunological response to an antigen. Adjuvants are necessary in many vaccine formulations in order to elicit the desired immune response from the patient. Normally, a certain class of immune cells known as Antigen Presenting Cells (APCs) present on their surface foreign molecules, peptides and proteins to T- and B-cells which develop antibodies and receptors specific to the presented molecule. Adjuvants work by alerting the immune system to respond to a certain antigen, and by facilitating this antigen presentation process. More focus is being placed on lectins as alternative adjuvants. The concept is relatively simple: lectins may facilitate a greater response to an antigen by delivering that antigen directly into the presentation pathways located within APCs. Plant lectins are attractive for filling the adjuvant role because they specifically target the mucosal areas where protection is needed the most. Work by Lavelle, et al. showed that the type II RIP mistletoe lectin I (MLI) is a strong mucosal adjuvant. The researchers co-administered several plant lectins with ovalbumin (OVA), the model antigen, to mice. As a control they used cholera toxin (CT), a strong model adjuvant. The researchers investigated tomato lectin (LEA), wheat germ agglutinin (WGA), Ulex europaeus lectin 1 (UEA-1) or MLI co-administered with OVA via an intranasal route. They found that only MLI and CT gave high titers of OVA-specific IgG and IgA, indicating that type II RIPS (such as ricin) may indeed be a suitable platform for a mucosal adjuvant technology. They also found that MLI itself was highly immunogenic, so obviously more work must be done to overcome this. More recent work by this group has shown that all three type II RIPS from mistletoe (MLI, MLII, MLIII) produce equivalent IgG and IgA titers against the herpes simplex virus glycoprotein D2 (gD2) as does CT. Additionally, mistletoe type II RIPS induced more of a T_H2-type immune response.
response is indicative of humoral immunity, or antibody, rather than cellular protection. Elevated levels of antigen-specific secretory IgA indicates that the response is occurring primarily at the mucosal surfaces, which is where pathogen attacks mostly occur. The major drawback of this system is the immunogenicity of ML itself. This immunogenicity may be due to the toxicity of ML. Ideally, one would prefer to have an adjuvant that does not induce an immune response against itself, as repeated administrations of such a formulation may induce adjuvant-specific antibodies which may reduce the efficacy of the vaccine, necessitating a higher dose to achieve the same effect.

Other groups are focusing their efforts on building an adjuvant system around ricin, the model type II RIP. A group of scientists working at the University of Warwick in the UK is focusing on utilization of a disarmed RTA molecule\(^\text{28}\) as a carrier of antigenic peptides. By creating genetic fusions of various peptides to the N-terminus of this RTA\(_{R180H}\), expressing this construct in \(E.\ coli\) and re-associating the purified fusion protein with castor bean-derived RTB, they have developed an interesting antigen delivery system. Smith et al. demonstrated that when a short peptide of the influenza nucleoprotein (NP) was used in their system and administered to RMA-S cells, NP-specific cytotoxic T-lymphocytes (CTL) were able to recognize and lyse them. CTLs specific for gp33, a different peptide, did not kill the treated cells. In addition, by inserting a potential signal peptidase recognition site between the NP peptide and RTA\(_{R180H}\), they showed that the presentation of NP on the cell surface was dependent on signal peptidase. These findings suggest that peptides to be presented on the surface via binding to Major Histocompatibility Complex I (MHC-I) proteins are being processed through the ER.\(^\text{29}\)

In the most recent work by this group, Grimaldi, et al. showed that a pneumovirus peptide (P\(_{261}\)) fused to the N-terminus of RTA\(_{R180H}\) stimulated interferon-\(\gamma\) (IFN-\(\gamma\)) production from P\(_{261}\)-
specific CD8\(^+\) T-cells, however no antibody response was observed. In these experiments, mice were administered with 100 ng of P\(_{261}\)-ricin intraperitoneally, and they report the immune response observed was consistent with presentation of the MHC class I type, indicating that this system takes advantage of the retrograde pathway (see Figure 3). Experiments in which vaccinated mice were challenged with pneumovirus demonstrated that the vaccine failed to protect the animals, however the onset of disease was delayed. Grimaldi, et al. also looked at the immune response towards ricin holotoxin, RTA, and RTB. Interestingly, they saw high levels of anti-RTA and anti-ricin antibodies but “minimal levels of anti-RTB antibodies”, suggesting that their system induces a strong response towards RTA, but not RTB.\(^{30}\) Although these experiments highlight the efficacy of ricin in mediating antigen presentation, their platform is limited by the potential toxicity due to the source of the RTB (castor bean) and residual RTA\(_{R180H}\) activity and the generation of significant antibody levels to the ricin carrier. The mutant used, RTA\(_{R180H}\), exhibits a reported 500-fold decrease in cytotoxicity\(^{28}\), however, the RTB that is used is derived from a castor bean source that must be separated away from wild-type RTA. For each application, this system must be validated by \textit{in vitro} N-glycosidase assays and \textit{in vivo} by cytotoxicity assays.\(^{31}\)

Our group is interested in utilizing the RTB lectin, as opposed to the ricin holotoxin, as a mucosal adjuvant. The advantage of this system is the total lack of RTA in any form, and it takes advantage of the low immunogenicity of RTB. Medina-Bolivar, et al. fused the green fluorescent protein (GFP) to the C-terminus of RTB and expressed this construct in transgenic tobacco hairy roots. The patatin signal peptide directed secretion of the RTB:GFP fusion protein to the growth medium, providing for a simpler purification regime. Intranasal administration of mice with RTB:GFP demonstrated an enhanced specific anti-GFP IgG titer over GFP alone or
GFP mixed with galactosamine-purified non-transgenic hairy root media fractions. The RTB-mediated immune response was comparable to that seen with GFP co-administered with cholera toxin (CT), a model mucosal adjuvant. Furthermore, the type of response observed based on IgG2a:IgG1 ratios was consistent with a T\textsubscript{H}2 type of immune response, and additionally they observed antigen (GFP)-specific secretory IgAs at the mucosal areas of RTB:GFP- and CT + GFP-treated mice.\textsuperscript{32} Unpublished data from our group demonstrates that an immune response is not observed when antigen (OVA) is simply mixed with RTB, i.e., not fused. Thus the “carrier/presentation” function may be the primary route of RTB-mediated immune enhancement. In contrast, the immune response observed by Lavelle, et al. using MLI holotoxin mixed with antigen may be due to the toxicity of MLI stimulating a response. Furthermore, it demonstrates that when using a non-toxic adjuvant such as RTB, immune response is dependent on a delivery mechanism.

A second group working in Loma Linda University headed by William Langridge is also focusing on using only RTB as an adjuvant. This group has expressed an outer capsid protein (VP7) of rotavirus-RTB fusion protein (VP7:RTB) in transgenic potato. The aim here was to produce an edible vaccine. Detectable levels (0.03% total soluble protein) of VP7:RTB was observed, and the VP7 fusion partner did not inhibit lectin binding of RTB, indicating that such a fusion does not sterically hinder RTB activity. However, as to date, this group has not investigated the immune response of VP7:RTB.\textsuperscript{33} This group has also been producing antigen:RTB fusion proteins in \textit{E. coli}, perhaps to overcome the low expression level of potato-derived RTB fusions. In these studies, they fused the 90 amino acid peptide NSP4 from simian rotavirus to the N-terminus of RTB and expressed it in \textit{E. coli}. All of the fusion protein was found in inclusion bodies, and therefore had to be refolded after denaturation with urea.
Refolded, purified NSP4:RTB administered intranasally to mice showed an increased response to NSP4 over heat-denatured NPS4:RTB and NSP4 alone.\textsuperscript{34} In addition, the researchers found that the response elicited was a $T_{H1}$ response, as determined by ratios of IgG2a to IgG1 and through cytokine profiles.\textsuperscript{35}

IV. Targeting lysosomal storage disorders

Lysosomal storage disorders are diseases in which there is an impairment of normal lysosomal function. Lysosomes are small cellular compartments that are characterized by low pH and the presence of high amounts of hydrolytic enzymes. Their purpose in the cell is to break down molecules that are toxic or need to be recycled, such as cell surface receptors. Due to this important role, and owing to the diverse types of molecules which are degraded in lysosomes, disruptions or mutations in a single gene which codes for a lysosomal enzyme can result in devastating and often fatal conditions. The classic and the most common example of a lysosomal storage disorder is Gaucher disease.

Gaucher disease results from mutations in the $\beta$-glucocerebrosidase gene. The three types of disease result from different mutations that range from lower $\beta$-glucocerebrosidase activity to complete loss of function. $\beta$-glucocerebrosidase catalyzes the breakdown of glucosylceramide to glucose and ceramide, and loss of function leads to a deadly accumulation of the substrate. Loss of $\beta$-glucocerebrosidase function impacts macrophages most, as this class of cells is important in clearing dead red and white blood cells from the system. The symptoms range from liver problems to bone disease to disfigurement and death.\textsuperscript{36}

Treatment options for Gaucher patients mostly revolve around enzyme replacement therapy (ERT). However, other options are being explored. They include gene therapy,
substrate reduction therapy and chaperone therapy. In gene therapy, the goal is to deliver working copies of the β-glucocerebrosidase gene, through stem cell and virus-mediated means, and seems to work well in some mouse models. Substrate reduction therapy focuses on inhibiting the synthesis of the substrate for β-glucocerebrosidase, barring the need for a fully functional enzyme. N-butyldeoxynojirimycin, an inhibitor of glucosylceramide synthesis has been available for use in sufferers of type I Gaucher disease since 2002 under the name Zavesca, without any major adverse effects. Although these technologies are promising, the most common treatment for Gaucher is ERT.

ERT using β-glucocerebrosidase relies on the presence of mannose-terminated glycans to be taken up by target macrophages, which display mannose receptors on their surface. We want to ask if uptake of lysosomal enzymes such as β-glucocerebrosidase can be facilitated via RTB-mediated delivery. It has been shown that β-glucocerebrosidase displaying the proper glycans can be made in plants. Coupling the effect of mannose receptor-mediated uptake with the action of RTB may reduce the dosage and thus the cost of therapy. A similar approach has recently been attempted, by fusing β-glucocerebrosidase to the HIV-1 trans-activator protein transduction domain (TAT), expressing this fusion in eukaryotic cells and administering to cultured cells. Cells which do not display mannose receptors readily endocytosed the fusion proteins. This indicates that a strategy which expands the variation in target cells may hold certain advantages in treating these types of disorders. Given that RTB delivers a significant amount of RTA to lysosomes during ricin poisoning, RTB would make a good choice as a delivery vehicle for lysosomal enzymes.
References


Figure 3, page 7, is adapted from an image created by Maureen Dolan, Ph.D., Research Assistant Professor of Arkansas Biosciences Institute (2003). Used by permission.
Chapter 2:
Plant-produced recombinant RTB can deliver chemically conjugated or genetically fused payloads into human cells

Abstract
The galactose/N-acetyl-galactosamine specific lectin ricin B-chain (RTB) from *Ricinus communis* can mediate cellular uptake of payload small molecules and proteins via chemical conjugation at primary amines, through the biotin/streptavidin interaction, and through direct genetic fusion. Recombinant RTB (rRTB) and RTB-fusions produced in an *Agrobacterium tumefaciens* mediated transient expression system in *Nicotiana benthamiana* exhibit comparable lectin activity and uptake functions to castor bean-derived RTB in human HT-29 cells. Expression levels using this system are 0.5 – 1.0% TSP. The recombinant form of RTB is an attractive source for ricin- and RTB-based carrier platforms, as it does not require processing of toxic material. A single RTB-specific purification protocol allows for efficient purification of rRTB and a wide variety of different RTB-fusions from infiltrated leaves, with ~ 60% recovery. Plant-based production of rRTB and RTB fusions is a flexible and efficient protein delivery system.
Introduction

Nature has evolved clever ways in which to deliver toxic proteins across the barrier of the cell membrane. Some of these toxins, such as the type II ribosome inactivating protein (RIP) ricin from the seeds of the castor bean plant (*Ricinus communis*), must navigate through labyrinthine paths once inside the cell to reach their substrate. In the case of ricin, it must reach the ribosomes which reside in the cytoplasm. I am interested in determining whether, by taking advantage of certain aspects of ricin function, such as the uptake and trafficking within target cells, while eliminating the toxic effects of ribosome inactivation, it may be possible to create a safe and effective means to deliver proteins to cells which otherwise are not able pass the outer membrane barrier.

Ricin toxin is a heterodimeric protein consisting of the 32 kD N-glycosidase A-chain (RTA) which mediates ricin toxicity and the 32 kD galactose/N-acetyl-galactosamine-specific lectin B-chain (RTB), connected via a single disulfide bond. Upon ingestion or inhalation of the toxin, ricin is taken up by cells through the binding of RTB to cell surface galactose residues. A large portion of endocytosed ricin remains in lysosomal and/or endosomal compartments. However, some of the ricin RTA is delivered to the cytoplasm via retrograde transport through the Golgi to the endoplasmic reticulum (ER). Once in the ER, RTA and RTB dissociate, and RTA is translocated to the cytoplasm via Sec61p-dependent pathways, where it presumably evades ubiquitination and ER-associated degradation through a low abundance of lysine residues. Only then is RTA given access to the 28 S ribosomal RNA, upon which it de-purinates a specific nucleotide, halting protein synthesis.
Researchers are exploring whether the trafficking of ricin within mammalian cells can be exploited for antigen or toxin delivery in immunological applications (see Figure 1). One promising area of research is the use of RTA in immunotoxins, conjugated to monoclonal antibodies specific to certain cancer cell types. Other groups are interested in exploiting the delivery potential of RTB to carry antigens or therapeutics across cell membranes. Early work by Roth, et al. and Hofmann, et al. demonstrated that insulin bound to RTB via disulfide bonds increased sensitivity in cell lines that were either sensitive or insensitive to insulin. However, the aim was not to deliver insulin as much as to facilitate binding to the cell surface to increase insulin:receptor interactions. More recently, Beaumelle, et al. showed that dihydrofolate reductase was transported to the cytosol (through an unfolded intermediate) of target cells when fused to the N-terminus of a disarmed RTA (RTA_{R180H}) and re-associated

![Figure 1. The various pathways a ricin-mediated antigen delivery system may exploit. Upon endocytosis, a large portion of ricin moves into the Lysosomal pathway, and cycles between lysosomal and endosomal compartments, as well as the cell surface. In Antigen Presenting Cells (APC) this pathway leads to Major Histocompatibility Complex II presentation. Some internalized ricin moves into the Retrograde pathway, traveling through the Golgi to the ER (via RTB's interaction with calreticulin), where RTA and RTB dissociate and RTA is transported to the cytosol by Sec61p. In APCs the retrograde pathway leads to MHC class I antigen presentation. In epithelial cells, the transcytosis pathway may lead to contact with and uptake by APCs. (Permission for use granted by Maureen Dolan, Ph.D., Arkansas Biosciences Institute)](image-url)
with castor bean-derived RTB (cbRTB). \textsuperscript{14} Tagge et al. performed similar experiments with green fluorescent protein (GFP) fused to RTA\textsubscript{R180H}, then reassembled with cbRTB, and was able to follow the trafficking in Hep3B and KB cells through fluorescence microscopy. \textsuperscript{15} Because antigen presentation through the Major Histocompatibility Complex I (MHC I) pathway also involves Sec61p transport to the cytosol, researchers have explored whether the ricin transport pathway could be exploited for vaccine strategies (see Figure 1). Grimaldi, et al. has shown that a short peptide of the mouse pneumovirus (P\textsubscript{261}) fused to RTA\textsubscript{R180H}, re-associated with RTB and delivered to mice intraperitoneally produced P\textsubscript{261}-specific CD8\textsuperscript{+} T-cells, but did not protect against viral challenge. Results reported by this group were consistent with MHC class I presentation. \textsuperscript{16}

RTB’s specificity makes it uniquely suited to mucosal vaccines. Mucosal surfaces comprise the site of most infection and therefore the location where strong immunity is required. Medina-Bolivar, et al. demonstrated the adjuvancy of RTB when fused to the model antigen GFP. RTB:GFP fusions were produced in transgenic tobacco hairy root cultures and administered to mice intranasally. RTB mediated the induction of strong GFP-specific immune responses comparable to that of cholera toxin B, a model mucosal adjuvant. \textsuperscript{17} However, in the course of these experiments, cellular uptake of RTB:GFP was not characterized. Work by Choi, et al. also used only RTB and focused primarily on vaccine applications, concentrating on RTB’s mucosal specificity. Researchers from this group have shown that an outer capsid glycoprotein (VP7) of simian rotavirus SA11 fused to the N-terminus of RTB is produced in potato with biological activity, but at low expression levels. \textsuperscript{18} They have also shown that the fusion of NSP4 peptide of rotavirus to RTB, expressed in \textit{E. coli} as inclusion bodies and refolded, enhanced the immunogenicity of NSP4 in mice over NSP4 alone. \textsuperscript{19} Unpublished data from our group has
shown that RTB does not exhibit adjuvancy effects when administered as an ad-mix (i.e. not genetically fused or otherwise conjugated to) with ovalbumin (OVA) in mice. In contrast, a stimulation of OVA-specific immune response was observed by OVA ad-mixed with mistletoe lectin I (MLI), a less toxic type II RIP. These data showed that toxicity plays a role in immune response. Given that RTB alone is non-toxic, I hypothesize that RTB’s adjuvancy as observed by Medina-Bolivar and Choi is due to a direct delivery of antigens to antigen presenting cells (APCs).

I therefore ask the question of whether a recombinant RTB (rRTB) can be broadly used as a platform for a flexible and efficient protein delivery system. Producing a recombinant form of RTB eliminates the requirement of separating RTB from RTA in ricin toxin, as the gene encoding RTA would never be present. This precludes the possibility of toxicity. Secondly, I ask if certain methods of attaching “payload” molecules and/or proteins to rRTB interfere with its lectin and cellular uptake functions. I report here: 1) the production of rRTB and RTB:GFP in Nicotiana benthamiana by Agrobacterium-mediated transient expression, and subsequent purification; 2) the fluorescein labeling and biotinylation of rRTB; and 3) the uptake of fluorescein-rRTB, rRTB-biotin complexed with fluorescein-streptavidin, and RTB:GFP in human epithelial HT-29 cells. This research shows that potential payloads can be connected to the RTB carrier through various ways, including chemical conjugation at primary amines and direct fusion of polypeptides. Also, payload molecules conjugated to streptavidin can be carried into cells via binding to biotinylated RTB in vitro.
Methods

Gene constructs

Maps of the constructs used in these studies are shown in Figure 2. The creation of construct R6-2, encoding RTB:GFP has been described elsewhere.\textsuperscript{17}

Sequences encoding RTB (rRTB) were PCR amplified using 5’-TCTAGAGCTGATTTCTATGGAT (F) and 5’-GTCGACTCAAAAATAATGGTAACCATA (R) using Pfu DNA polymerase (Invitrogen, Carlsbad CA). The template used was R6-2. These primers added the XbaI restriction site on the 5’ end of the gene (underlined), and a stop codon (TGA; in red) and SalI site on the 3’ end (bold). The HindIII/XbaI fragment containing the dual enhanced cauliflower mosaic virus (CaMV) 35S promoter, the tobacco etch virus (TEV) translational enhancer, and the patatin signal peptide (de35S:TEV::sp) was isolated from plasmid pBC-R6-2. The de35S:TEV::sp fragment and the rRTB PCR product were ligated into the pBC cloning vector (Stratagene, Cedar Creek TX) which was digested with HindIII and SalI in a tri-molecular reaction to give plasmid pBC-35S::rRTB. After sequence confirmation, the promoter:gene cassette was subcloned into the pBlB-Kan\textsuperscript{20} binary vector via HindIII/SalI.

![Figure 2. Recombinant RTB (rRTB) and RTB:GFP gene construct maps. Both constructs are driven by the constitutive dual enhanced 35S CaMV promoter and contain the patatin signal peptide (“sp”). Constructs were assembled in pBC and promoter:gene cassettes were sub-cloned into the pBlB-Kan binary vector (via HindIII/SalI for rRTB and HindIII/SalI for RTB:GFP).](image-url)
Plant growth conditions

Seeds of *Nicotiana benthamiana*, provided by Dr. S. Tolin (Virginia Tech, Blacksburg VA), were germinated by direct-seeding into 4 inch pots and used for expressing rRTB and RTB:GFP. The growth media used was a 2:1 mixture of Promix BX and PGX (Hummert). Growth conditions of 16 hr photoperiod (180 \( \mu \text{mol s}^{-2} \text{ m}^{-1} \)), 25°C days, 21°C nights, 65% humidity were maintained via Conviron ATC60 growth chamber. Plants were watered as needed. Plants 5 – 6 weeks from seeding were selected for infiltration. The typical yield of infiltrated material was 10 – 20 g fresh weight per plant.

*Agrobacterium tumefaciens*-mediated transient expression

pBIB-Kan plasmids harboring promoter:gene cassettes were transformed into *A. tumefaciens* strain LBA4404 using a modified freeze/thaw method. Positive clones were grown in 50 mL YEP medium (10 g/L bacto-peptone, 10 g/L yeast extract, 5 g/L NaCl) containing 100 \( \mu \text{g/mL} \) kanamycin and 60 \( \mu \text{g/mL} \) streptomycin for 48 hr at 28°C, 220 rpm. To induce *A. tumefaciens* prior to infiltration, cell pellets were harvested via centrifugation (5000 X g for 10 min), resuspended in 300 mL induction media (20 mM MES pH 5.5, 0.3 g/L MgSO\(_4\) \( \cdot \) 7H\(_2\)O, 0.15 g/L KCl, 0.01 g/L CaCl\(_2\), 0.0025 g/L FeSO\(_4\) \( \cdot \) 7H\(_2\)O, 2 mL/L 1 M NaH\(_2\)PO\(_4\) pH 7.0, 10 g/L glucose) containing 100 \( \mu \text{g/mL} \) kanamycin and 60 \( \mu \text{g/mL} \) streptomycin, supplemented with 0.2 \( \mu \text{M} \) acetosyringone and incubated at 28°C, 220 rpm, for 4 hr to overnight. Induced *A. tumefaciens* cultures were introduced into four to six week old *Nicotiana benthamiana* plants either by pressure injection or vacuum infiltration. For pressure injection, a disposable syringe without a needle was filled with *A. tumefaciens* culture and pressed against the underside of the leaf. For vacuum infiltration, plants were place upside-down in a beaker containing the
induced culture so that all aerial portions were submerged. This was then placed inside a vacuum chamber and vacuum was applied (approximately 1 min) and broken by abruptly pulling off the tube from the chamber. This procedure was performed twice for each plant to ensure complete infiltration. Following infiltration, plants were replaced to their growth chambers and allowed to incubate for 48 – 72 hr.

Extraction and purification of RTB and RTB:GFP fusion proteins

Agrobacterium-infiltrated leaves, 10 – 20 g, were ground under liquid nitrogen (LN₂) in a mortar and pestle to a fine powder. Extraction buffer 2 (50 mL of 100 mM Tris-HCl pH 7.5, 20 mM D-galactose, 1% PVPP) was added to the powder and allowed to thaw at RT. The resulting crude extract was centrifuged at 14,200 X g for 30 min at 4°C. The supernatant was filtered through KimWipes, brought to 100 mL with distilled H₂O and the pH was adjusted to 7.5 with 1 N NaOH. This cleared extract was then filtered through a 0.45 μm membrane and loaded onto an equilibrated 20 mL column volume MacroPrep High Q (Bio-Rad, Hercules CA) column using a Bio-Rad Duo-Flow FPLC system. Following loading of the sample, the column was washed with 80 mL of 50 mM Tris-HCl pH 7.5. The RTB-containing proteins were eluted and collected from the column by washing with 45 mL 50 mM Tris-HCl pH 7.5, 400 mM NaCl. The column was then cleaned by washing with 50 mM Tris-HCl pH 7.5, 1 M NaCl and re-equilibrated with 50 mM Tris-HCl pH 7.5. The RTB-containing sample (400 mM NaCl) was loaded onto a 1 mL immobilized lactose column (EY Laboratories, San Mateo CA) and washed with PBS (37.5 mM Na₂HPO₄, 12.5 mM NaH₂PO₄, 150 mM NaCl, pH 7.0). Purified RTB and RTB-containing fusion proteins were eluted by washing with 4 X 1 mL PBS + 500 mM D-galactose. RTB-containing samples were then concentrated using YM-10 Centricons (Millipore Corp., Bedford
MA) and dialyzed to PBS. Concentrated, dialyzed samples were then analyzed via silver stained SDS-PAGE and asialofetuin binding assay. SDS-PAGE was performed using 10% or 12% PAGE-gels (PAGE-gel, Inc. San Diego, CA). Silver staining was performed using the SilverSnap kit (Pierce, Rockford, IL).

Asialofetuin binding assay.

A functional ELISA utilizing asialofetuin instead of a capture antibody was employed to assess galactose-specific lectin activity and quantify rRTB and RTB-containing fusion proteins. Asialofetuin is a modified mammalian glycoprotein that contains galactose-terminated glycans (Sigma, St. Louis MO). Asialofetuin at 300 µg/mL in PBS was bound to the wells of an Immulon 4HBX plate for 1 hr at RT. The wells were then blocked with 3% BSA in PBS for 1 hr at RT. Castor bean-derived RTB (cbRTB; Vector Labs, Burlingame CA) was used for the standard curve, ranging from 1.95 to 250 ng/well in PBS + 10 mM D-galactose. For asialofetuin binding, 100 µL of standards and samples incubated at RT for 1 hr. The plate was then washed 3X with PBS (300 µL/well). Rabbit anti-\textit{Ricinus communis} lectin antibody (Sigma R-1254), diluted to 1:4000 in blocking buffer was then added (200 µL/well) and allowed to incubate for 1 hr at RT. Wells were washed again and alkaline phosphatase labeled goat anti-rabbit antibody (Bio-Rad, Hercules CA), diluted 1:4000 in blocking buffer, was added and allowed to incubate for 45 min at RT. The wells were washed a third time and alkaline phosphatase substrate (100 µL/well; Pierce, Rockford IL) was applied. After the color developed sufficiently (10 – 15 min), the reaction was stopped with the addition of 50 µL 2 N NaOH and the absorbance at 405 nm was recorded. The inverse of the absorbance at 405 nm was plotted vs. the inverse of the
standard RTB/well to give a linear relationship. This equation was then used to estimate the amount of RTB in the samples in terms of RTB equivalents.

By probing with antibodies or proteins other than the rabbit anti-*Ricinus communis* lectin antibody used for quantification, it was possible to use this assay to determine carrier/payload interactions. For example, cbRTB and rRTB samples that underwent biotinylation chemistry (see below) were assessed for lectin activity and confirmation of biotinylation by probing with horseradish peroxidase-labeled streptavidin (Sigma, St. Louis MO) instead of anti-ricin antibodies. By inclusion of the proper controls, a positive reaction in this scenario indicated that biotin must be present since binding to asialofetuin presumed the presence of RTB. Identical samples probed with anti-ricin antibodies confirmed this.

**Production of RTB specific antibodies**

The 789bp fragment encoding the RTB portion of ricin toxin was amplified from preproricin template using primers 5’-CATATGGCTGATTTTGTATGGATC (F) and 5’-GTCGACTCAAAATAATGGATAACCATA (R) to add *Ndel* (underlined) to the 5’ end and *SalI* to the 3’ end (bold). A stop codon (red) was added just upstream of the *SalI* site. This fragment was cloned into pET41 (EMD Biosciences, San Diego CA). The use of the *Ndel* site at the 5’ end removed the vector-encoded GST sequences, creating a RTB gene containing no vector-encoded tags. pET-RTB was transformed into *E. coli* strain BL21(DE3). A 5 mL overnight culture was used to inoculate 1 L LB containing 100 µg/mL kanamycin (2 L flask). The culture was grown at 37°C (220 rpm) for ~3 hr or until the OD$_{600}$ reached ~ 0.8. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and allowed to incubate at 37°C (220 rpm) for 3 hr. The cells were harvested via centrifugation and lysed in 30
mL 1X BugBuster (EMD Biosciences) reagent supplemented with 1 mg/mL lysozyme, 10 µg/mL DNase I, 10 µg/mL RNase A. The crude extract was centrifuged at 13,000 X g for 15 min. Inclusion bodies were recovered; and there was no detectable RTB in the soluble fraction via Coomassie stained SDS-PAGE analysis of pre- and post-induction fractions. This is consistent of other reports of E. coli produced RTB. Inclusion bodies were washed 3X with 25 mL 50 mM Tris-HCl pH 8.0, 0.1% Triton X-100, then dissolved in 2 mL 50 mM CAPS pH 11.0, 0.3% N-laurylsarcosine, 5 mM DTT. This was dialyzed to PBS + 5 mM DTT 2X at 4°C. The solubilized RTB migrated to the predicted molecular weight on SDS-PAGE (see Figure 3a) but showed no activity via asialofetuin assay or immobilized lactose chromatography. This is most likely due to misfolding.

As an aside, it should be noted here that many attempts were made to optimize E. coli-derived rRTB, in terms of activity of refolded protein. No reports are known that have shown production of soluble rRTB in E. coli. Protocols reported by Tonevitsky, et al., and Choi, et al., as well several other methods not reported were tried, with limited success. Different chaotropic reagents such as urea, guanidine HCl, and N-laurylsarcosine were tested. Methods such as dilution and successive rounds of dialysis to remove the chaotropic reagent after solubilization of the inclusion bodies, and therefore induce refolding, were also tried. The addition of redox pairs such as reduced/oxidized glutathione and cysteine/cystine, as well as reductants such as DTT and βME also proved inefficient. The presence of galactose in the refolding buffer also did not produce significant quantities of active rRTB, in our hands. I therefore only used E. coli-derived rRTB for the production of antibodies.

Approximately 1 mg of solublized inactive E. coli-derived RTB (Figure 3a) was sent to CoCalico Biologicals (Reamstown PA) for raising an RTB-specific antibody in rabbit. Rabbit
serum was tested for reactivity towards plant-derived RTB (cbRTB and 6HIS-RTB, a gift of Dr. Medina-Bolivar, described elsewhere\textsuperscript{26}) via western blots (see Figure 3b). Total IgG was collected from the pooled positive sera via Protein A chromatography. After removing unbound material with PBS, bound IgG was eluted from Protein A sepharose beads (Sigma) by washing with 100 mM glycine pH 3.0 which was immediately neutralized with 1 M Tris-HCl pH 9.5. IgG fractions were pooled and concentrated via YM-30 Centricons (Millipore Corp., Bedford MA). Glycerol was added to a final concentration of 50\% and antibodies were stored at −20°C. The titer for standard western blots is as high as 1:20,000 with sensitivity as low as 1 ng/lane.

Fluorescein labeling

cbRTB and purified rRTB were labeled at primary amine groups with NHS-Fluorescein (Pierce 46100) as per the manufacturer’s instructions. The labeling reaction was allowed to proceed for 2 hr at RT. Unreacted NHS-fluorescein was quenched by the addition of Tris-HCl pH 7.5 to a final concentration of 50 mM. The reaction mixture was then dialyzed to PBS 2X at 4°C.
Biotinylation of RTB and binding to streptavidin

cbRTB and rRTB were labeled at primary amine groups with sulfo-NHS-LC-biotin (Pierce 21335) as per the manufacturer’s instruction. The labeling reaction was allowed to proceed for 2 hr at RT. Unreacted sulfo-NHS-LC-biotin was quenched by the addition of Tris-HCl pH 7.5 to a final concentration of 50 mM. The reaction mixture was then dialyzed to PBS 2X at 4°C. Biotinylation of RTB was confirmed via a modified asialofetuin assay. Briefly, RTB-biotin was applied to microtiter plate wells that were coated with asialofetuin and then blocked. This was allowed to incubate at RT for 1 hr, then washed with PBS. Horseradish peroxidase-labeled streptavidin (strep-HRP) was then applied to wells and allowed to incubate at RT for 20 min. Horseradish peroxidase (HRP) substrate (KPL, Gaithersburg MD) was added after washing and the color was allowed to develop for ~10 min before stopping with 1 N H$_2$SO$_4$. The absorbance at 450 nm was then read. Only wells containing biotinylated RTB gave a reaction. The extent of biotinylation on a molar ratio basis was not determined. Prior to uptake, cbRTB-biotin and rRTB-biotin were mixed with fluorescein-labeled streptavidin (Sigma S-3762).

Cell uptake and fluorescence microscopy

For experiments to test the cellular uptake function of the various carrier(payload) schemes developed, I used HT-29 human epithelial cells (ATCC) grown to 50 – 75% confluence, as estimated via visual inspection, in individual wells of an optical-bottom 96-well-microtiter plate (#165305; Nalge Nunc International, Rochester NY) in McCoy’s 5A media (+ 5% fetal bovine serum; Invitrogen, Carlsbad CA). Prior to sample loading, cells were washed 3X with ice-cold Hank’s Balanced Salt Solution (HBSS; Invitrogen). Samples for uptake were brought to
100 µL with either HBSS or PBS before adding to the cells. Samples and cells were incubated at 4°C for 30 min to allow binding to the cell surface without being endocytosed. Cells were then washed 3X with ice-cold HBSS again to remove any unbound proteins. Ice-cold HBSS (200 µL/well) was then added, and the T = 0 picture taken. Fluorescence microscopy was performed using a Zeiss Axiovert 200M microscope fitted with SensiCam QE digital camera, and utilized IP Lab software (Carl Zeiss Inc.). Photographs were taken at 40X magnification using a GFP Endow GFP BP filter set (Chroma Technology Corp., Brattleboro VT). The plate containing the cells was then incubated at 37°C, 5% CO₂ (normal growth conditions) to initiate endocytosis. Additional pictures were taken at T = 30, 60 and 120 min.

**Results**

**Expression and purification of rRTB and RTB-GFP**

In order to have a flexible source of rRTB and RTB-fusions, an *Agrobacterium*-mediated transient expression system using *Nicotiana benthamiana* was optimized. Efforts to develop a bacteria-based rRTB production system did not yield sufficient lectin-active rRTB. rRTB produced in *E. coli* was almost completely found in insoluble inclusion bodies. Re-naturation of this insoluble rRTB to an active form was inefficient (see Methods), and the feasibility of such a system on a commercial scale is low. The ideal production system would be a plant-based one, since RTB is a plant protein. In addition, bacterial systems such as *E. coli* do not have the capability to perform post-translational glycosylation. It has been reported by Frankel, et al. that the mannose-containing glycans on RTB facilitate uptake through the cell-surface mannose receptor in the absence of RTB lectin activity.\(^{27}\) This suggests that presence of the glycan is important in providing yet another route for RTB to enter the cell and deliver payload proteins.
To the author’s knowledge, no type II RIP has been discovered in *Nicotiana* spp., suggesting that rRTB produced in *N. benthamiana* is unlikely to interact with an endogenous type II RIP A-chain.

By infiltrating *Agrobacterium tumefaciens* cultures directly into the leaves of *N. benthamiana*, a short (up to 5 – 7 days) burst of transient expression of genes residing in the T-DNA portion of plasmids carried by the *A. tumefaciens* occurs. This short expression window allows testing of a large number of gene constructs in a short time, relative to stable transformation. Due to this flexibility and the observation that expression levels of transiently expressed genes were higher than the same gene stably transformed, I chose to utilize this *Agrobacterium*-mediated transient expression of rRTB and RTB-fusions in *N. benthamiana*.

Infiltrated leaves were harvested after 72 hr and subjected to extraction and RTB purification based anion exchange and lactose affinity chromatography (see Methods). Purity was assessed by observation of the proteins on silver stained 10% SDS-PAGE gels. There were little to no other bands seen, indicating a high level of purity. Plants infiltrated with empty-vector (pBIB-Kan) *Agrobacterium* and subjected to the exact same purification regime showed no bands on silver stain, indicating the specificity for RTB of this strategy (see Chapter 3, Figure 7). Identity of rRTB was confirmed by probing with RTB-specific antibodies, and by N-terminal sequencing (see Chapters 3 & 4). The level of expression of rRTB and RTB:GFP, as quantified via asialofetuin binding and Bradford assays was approximately 1.0% and 0.5% total soluble protein (TSP), respectively. The RTB-specific purification protocol typically produced 1 – 10 µg of purified protein per gram of leaf fresh weight. Figure 4 is a silver stained 10% SDS-PAGE gel showing the purity of recombinant rRTB, rRTB-fluorescein, rRTB-biotin + fluorescein-streptavidin, and RTB:GFP.
Fluorescein labeling and biotinylation of cbRTB and rRTB

To ask if attaching small payload molecules to RTB at primary amine groups affected lectin activity, cbRTB and rRTB were labeled with NHS-fluorescein. N-hydroxysuccinimide (NHS) reagents react with exposed primary amine groups (at the N-terminus and lysine side-chains), to create an amide bond between the protein and the molecule to which NHS is esterified to, in this case fluorescein. RTB theoretically contains eight primary amines: one at the N-terminus, and seven lysine residues. Following the reaction and two rounds of dialysis against PBS, the fluorescein-labeled protein samples retained a distinct yellow color, indicative of successful labeling. cbRTB-fluorescein and rRTB-fluorescein produced a brilliant green color when subjected to UV light. The lectin binding activity of cbRTB-fluorescein and rRTB-fluorescein was tested via asialofetuin binding assay. These proteins did not exhibit significant reduction in binding capability compared to unlabeled RTB. These results suggest that labeling RTB through the primary amine groups does not interfere with lectin activity. In addition, there was no discernable difference in asialofetuin binding between rRTB and cbRTB, indicating that recombinant production of RTB does not impact its activity. SDS-PAGE analysis of rRTB-fluorescein shows a slightly slower mobility than unlabeled rRTB (Figure 4, lanes 1 & 2). This difference in mobility is attributable to successful labeling of rRTB with NHS-fluorescein.
The streptavidin/biotin interaction was employed in order to determine if large proteins attached to RTB at primary amine groups interfere with lectin activity and cellular uptake. Streptavidin is a homo-tetramer with a molecular weight of about 67 kD. Each monomer binds one molecule of biotin, molecular weight 244.3 g/mol. This binding is one of the strongest known non-conjugated interactions, and it provides an easy way to attach a relatively large protein to RTB without creating a direct genetic fusion, by conjugating biotin to RTB then allowing streptavidin to bind the biotin. Biotinylation of cbRTB and rRTB was performed as described above using sulfo-NHS-LC-biotin, a water-soluble form of NHS-biotin that includes an 11 atom spacer arm (LC) between the NHS and the biotin, to reduce steric hindrances. The conjugation chemistry is the same as that described for the fluorescein labeling. A modified asialofetuin assay in which samples were probed with streptavidin-labeled horseradish peroxidase (HRP) was employed to assess the success of biotinylation. Comparing data obtained with this assay to an asialofetuin assay in which the same samples were probed with anti-ricin antibodies indicated that biotin was present on RTB, and that this RTB retained lectin activity. Unlabeled RTB, from both castor bean and recombinant sources and bound to asialofetuin, did not react with streptavidin-labeled HRP.
Both cbRTB-biotin and rRTB-biotin bound to asialofetuin reacted strongly with streptavidin-HRP as visualized with HRP substrate at 450 nm. These findings show that cbRTB-biotin and rRTB-biotin retain both lectin activity and streptavidin binding ability. The extent of biotin labeling on a molar ratio was not investigated. cbRTB-biotin and rRTB-biotin were complexed with fluorescein-labeled streptavidin, (FITC-streptavidin; Sigma S-3762, dissolved in PBS) by mixing equal amounts of both proteins and incubating at room temperature for ~ 20 min. As seen in Figure 4 lane 3, SDS-PAGE analysis of rRTB-biotin/ FITC-streptavidin complex shows several higher molecular weight bands indicative of the complex, as well as a light band corresponding to uncomplexed rRTB-biotin, which runs slower than rRTB. The gel running conditions undoubtedly break up some of the complex, as can be seen by the large ~20 kD band corresponding to streptavidin monomers. As this sample did not undergo purification, and FITC-strep was in excess in the mixture, there was some FITC-strep that did not complex with rRTB-biotin, which appears as the ~70 kD band.

**RTB mediates cell uptake of conjugated and fused payloads**

Fluorescent microscopy is a powerful tool that allows investigation of several questions regarding RTB-mediated uptake. First, it allows visual observation of fluorescently-labeled RTB in real time to assess the impact of conjugation and fusion on cell uptake. Second, comparison of rRTB to cbRTB indicates the impact of recombinant versus native expression on uptake.

The assay employed to investigate answers to these questions utilized human epithelial HT-29 cells grown in a thin layer on the inner surface of a 96 well optical-bottom, black-walled microtiter plate. In preparation for sample loading, the cells were cooled by washing with ice-cold HBSS to slow the cell surface activity. Ice-cold samples containing RTB were applied and
the plate was incubated at 4°C to allow binding to the cell surface yet halting of endocytosis. After this incubation, the cells were washed again with ice-cold HBSS to wash away any unbound protein. Photographs taken at this T = 0 time point usually show fluorescence only at the outer membrane, appearing as a complete outline of the cell. No internal structures are seen at this time. Incubation at 37°C allows endocytotic processes to recommence, and uptake of the fluorescent-labeled protein was visualized in real time by taking photographs at specific time points (T = 30, 60, and 120 min) following the shift to 37°C. If endocytosis and entry of labeled proteins into the endomembrane system occurs, this is typically observed as a reduced fluorescence at the plasma membrane and appearance of internal punctate fluorescence (presumably endosomes and lysosomes) at 60 and 120 min.

The various treatments tested in these experiments are listed in Table 1.

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>Proteins tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>cbRTB-fluorescein</td>
</tr>
<tr>
<td>2</td>
<td>rRTB-fluorescein</td>
</tr>
<tr>
<td>3</td>
<td>cbRTB + FITC-streptavidin</td>
</tr>
<tr>
<td>4</td>
<td>cbRTB-biotin + FITC-streptavidin</td>
</tr>
<tr>
<td>5</td>
<td>rRTB-biotin + FITC-streptavidin</td>
</tr>
<tr>
<td>6</td>
<td>GFP</td>
</tr>
<tr>
<td>7</td>
<td>RTB:GFP</td>
</tr>
</tbody>
</table>

Treatments 1 and 2 are fluorescein labeled cbRTB and rRTB, respectively. As seen in Figure 5, treatments 1 and 2 exhibited identical patterns of fluorescence as described above. Treatment 3 was non-biotinylated cbRTB, a negative control included to ensure that FITC-streptavidin was not capable of mediating uptake on its own. This is confirmed by absence of fluorescence at T = 0. Treatments 4 and 5 were biotinylated cbRTB and rRTB, respectively, preincubated with FITC-streptavidin. As shown in Figure 5, both showed positive fluorescence patterns over the time course, indicating the RTB mediated uptake of the fluorescent-labeled
streptavidin. Treatment 6 was GFP alone, a negative control. Lack of fluorescence in treatment 6 indicated inability of GFP to bind to cell surfaces on its own. A positive fluorescence pattern in treatment 7, RTB:GFP, indicated RTB-mediated uptake of fused GFP. HT-29 cells stained only with LysoTracker-Red (Molecular Probes, Eugene OR) show a very similar pattern (see Chapter 3), indicating that RTB locates primarily to endosomal and lysosomal compartments.
Discussion

I have reported the development of an Agrobacterium-mediated transient expression system using *N. benthamiana* to produce rRTB and RTB:GFP. Additionally I have shown that conjugation of proteins and small molecules to rRTB at primary amine groups does not adversely
affect lectin activity and cellular uptake function. RTB has been expressed in other systems previously, such as *E. coli*\textsuperscript{24}, *Saccharomyces cerevisiae*\textsuperscript{28}, *Xenopus* oocytes\textsuperscript{29}, *Spodoptera frugiperda* (Sf9) cells\textsuperscript{30,31}, monkey kidney COS cells\textsuperscript{32}, and tobacco\textsuperscript{26}. Additionally, ricin holotoxin has been produced in transgenic tobacco\textsuperscript{33}, and RTB:GFP has been produced in transgenic tobacco hairy root cultures.\textsuperscript{17} To our knowledge, RTB has not been produced in *Nicotiana benthamiana* using *Agrobacterium*-mediated transient expression. Work by Reed, et al. expressing hexahistidine-tagged RTB (6HIS-RTB) in stably-transformed tobacco reported an expression level of 0.007% TSP\textsuperscript{26}, in contrast to 1.0% TSP for rRTB in our system. Also, 6HIS-RTB transgenic lines produced at least three bands identified as RTB, due to alternative glycosylation forms and truncation at the N-terminus.\textsuperscript{26} rRTB from our system was purified as a single band and possessed mannose-containing glycans (approximately 85% of a RTB-fusion bound to Concanavalin A-sepharose, data not shown).

Ricin-based vaccine strategies such as the work of Grimaldi, et al. require RTB to associate with antigen-fused RTA\textsubscript{R180H}. The RTB used by this group is derived from castor bean, and therefore requires extensive purification to eliminate RTA and associated toxicity. This strategy has significant limitations for clinical applications both because of the potential of residual toxicity and the challenges of producing and processing highly toxic material at large scale. Absolute assurance of absence of RTA is guaranteed by producing RTB alone, recombinantly. In addition, the extensive tests involved in testing toxicity of payload-fused RTA\textsubscript{R180H}:RTB conjugates is tedious and expensive.\textsuperscript{34} It is doubtful that the public would accept a system based on RTB derived from ricin holotoxin, not to mention the costs such a system would incur to ensure safety and efficacy. rRTB produced in our system may in fact serve as a source for RTB in strategies such as that reported by Grimaldi et al. In our
experiments, I did not attempt to associate rRTB with RTA to create ricin, as I do not have a source for the “disarmed” RTA$^{R180H}$. However, I am optimistic that our rRTB can successfully interact with RTA to form ricin. Furthermore, by producing rRTB and RTB-fusions in a plant system such as Agrobacterium-infiltrated N. benthamiana, all recovered RTB species are active and soluble, unlike rRTB produced in E. coli, which must be refolded with varying degrees of success.

The Agrobacterium-mediated transient expression of transgenes in N. benthamiana is a very powerful tool. Many different genes and fusion constructs can be tested in a short amount of time compared to stable transformation of the same constructs. Additionally, the nature of the system is such that the expression profiles of infiltrated genes do not exhibit significant variation from plant-to-plant. By contrast, stable transformation of a single gene construct results in massive variations in expression levels among different plants transformed with the same construct, presumably due to so-called position effects, caused by the physical position of the transgene in the genome. The transient system utilizes a high Agrobacterium to plant cell ratio, and in contrast to stable transformation protocols, a higher number of inserted T-DNAs is desirable. The expression of the transgene is ectopic and transient in nature, and therefore the higher the number of T-DNAs to some saturation point, the greater the expression during the transient window. In stable transformation, one copy of inserted transgene per genome is desired, for silencing and genetics handling reasons. Both RTB:GFP and 6HIS-tagged rRTB are expressed at higher levels in transient systems compared to stable lines (data not shown), presumably for these reasons.

The purification method employed, anion exchange on High Q followed by lactose affinity chromatography, proved to be very versatile in terms of time, effort, and specificity for a
wide range of RTB-fusions. The regime allows for usable (µg - mg) amounts of rRTB or RTB-fusions to be extracted and purified in a single day. Using rRTB as a model, I estimate that the purification protocol yields ~ 60% recovery. A broad range of diverse RTB-fusions, such as RTB:GFP, IL12:RTB (see Liu & Cramer, 2006), RTB:κLC, and C1:RTB (see Chapter 3) have been purified using the exact same conditions. This flexibility enables rapid accumulation of purified RTB-fusion proteins, as specific purification conditions as influenced by each fusion partner may not have to be determined. This strategy complements the utility of the transient expression system and provides for a more user-friendly technology. In addition, this strategy is useful in investigating breakdown of certain RTB-fusions, as all breakdown products that contain an active RTB component are co-purified (see Chapter 4).

Conjugation of payloads to RTB at the primary amine groups did not significantly affect RTB-mediated binding to galactose or uptake by HT-29 cells. RTB contains eight possible sites of conjugation using this chemistry, at the N-terminus and seven lysine residues per polypeptide. Comparison of the bands in Figure 4 lanes 1 and 2 indicate that labeling of the RTB by NHS-fluorescein is complete, however it is not apparent how many labels per RTB are present. The band in lane 2 is completely shifted upward compared to the band in lane 1. If a mixture of different ratio label-to-RTB species were present, one would expect to see a band in lane 2 that lined up with the bottom of the band in lane 1, but would be higher at the top. Instead, what is seen is that the bottom of the lane 2 band is higher than the bottom of the lane 1 band. This suggests that perhaps only one or perhaps a few different ratios of label-to-RTB species are present, which in turn suggests efficient labeling at all exposed sites. The necessary mass-spectrophotometry experiments to confirm this have not yet been done. Uptake of FITC-streptavidin by biotinylated RTB showed that large proteins are able to be carried across the cell
membrane in a RTB-mediated manner. The availability of primary amine groups on RTB suggest that ratios of FITC-streptavidin to biotinylated RTB may be greater than 1, indicating that RTB can deliver conjugated proteins at least 67 kD in size, and perhaps multiple copies of the same protein. In terms of usage as a vaccine delivery system, this method may be able to deliver up to eight copies of antigen per RTB molecule, which may drastically enhance the immunogenicity and efficiency of such a system. However, the costs of a system that relies on chemical conjugation may be prohibitive in a clinical setting. The analyses required to determine the extent of FITC-streptavidin-to-biotinylated RTB interactions have not yet been performed.

Direct fusion of RTB to payload, in this case GFP, showed that this method of delivery is also efficient. As mentioned earlier, visual observation of RTB:GFP uptake was not performed in earlier reports, and uptake was assumed as a prerequisite of immune response. There are two potential drawbacks to strategies involving direct fusion. One involves the requirement of unique development for each fusion, typically requiring variants including N- and C-terminal arrangements, use of linkers, etc. A second major drawback to direct genetic fusion is the strength of the bond between carrier and payload. In a chemical conjugation scenario, it is possible to engineer breakable linkers between carrier and payload, for example through the use of sulfo-NHS-SS-biotin, a commercially available (Pierce) reagent that includes a disulfide bond between the NHS and biotin moieties. A situation in which it is desirable to have carrier and payload dissociate once inside the cell is plausible, however direct fusion of carrier and payload polypeptides would require a proteolytic step to realize this. An alternative is to explore different interactions between carrier and payload that do not involve chemical conjugation or direct genetic fusion. I have addressed this issue in Chapter 3.
References


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Figure 1, page 20, is adapted from an image created by Maureen Dolan, Ph.D., Research Assistant Professor of Arkansas Biosciences Institute (2003). Used by permission.
Chapter 3:

Engineering and evaluation of a RTB-mediated capture and carry system for effective delivery of payload proteins

Abstract

In order to improve the RTB-mediated delivery system to allow for sulfhydryl-linked payloads to the RTB carrier and to create a “capture and carry” platform, two strategies relying on known protein-protein interactions were tested. In the first strategy, the ability of the C-terminal domain of RTA to mediate interactions with RTB when fused to a payload protein was evaluated. It was observed that this domain is not sufficient for binding to RTB, when payloads were produced in both *E. coli* - (using a plant-derived RTB carrier) and plant-based (co-expressed with RTB carrier) protein production systems. The second strategy involved the engineering of an immunoglobulin heavy- and light-chain domain scaffold between carrier and payload. Several different plant produced RTB/immunoglobulin domain gene fusions and arrangements were tested. The selected carrier, RTB:κLC, did not interact with or bind to *E. coli*-derived Fd proteins. However, Fd:GFP co-expressed in leaves with RTB:κLC was co-purified using a RTB-specific extraction and purification protocol, but not when co-expressed with rRTB. Furthermore, the interaction between RTB:κLC and Fd:GFP included a disulfide bond, and RTB:κLC mediated uptake of Fd:GFP in HT-29 cells. These data show that *Agrobacterium*-mediated co-expression of RTB:κLC and Fd:payload proteins in *Nicotiana benthamiana* provides an efficient and flexible system for the production, purification, and application of RTB-mediated protein delivery.
**Introduction**

In Chapter 2 I demonstrated that attachment of small molecule and protein payloads to a recombinant ricin B-chain (rRTB) carrier through chemical conjugation at primary amine groups, biotin/streptavidin interactions or direct genetic fusion resulted in a flexible and efficient platform for delivery across outer cell membranes. Current research involves the improvement of the RTB-based carrier system by the development of a “capture and carry” coupling mechanism between carrier and payload. Ideally, one would want to attach payload proteins to the carrier through a breakable interaction. Allowing the payload and carrier to separate once inside the cell may give more flexibility and therefore a wider range of possible applications. Disulfide bonds are broken under very specific conditions, either through chemical reduction or enzyme mediated mechanisms. The major drawback of direct genetic fusion of carrier and payload proteins is the strength of the peptide bond, which usually requires a protease and an appropriate recognition sequence between fusion partners. Identifying the proper protease endogenous to the target cell that is located in the appropriate sub-cellular compartment would be tedious and may necessitate novel research. Breakable interactions such as the disulfide bond may be engineered into the spacers integrated into chemical conjugation reagents, but systems involving chemical conjugation may be hampered by the cost of such a system on a large scale. Other drawbacks of chemical conjugation include difficulties presented by the specific chemistry of payload proteins (for example, lack of available conjugation sites) and by the lack of control on conjugation efficiency.

The major goal of this research is to find an effective and easy way to attach payload proteins to RTB via a disulfide bond. I have chosen to investigate two possible ways to achieve such a coupling mechanism: Strategy I deals with exploitation of RTA structural domains
involved in dimerization with RTB, and **Strategy II** involves the utilization of an immunoglobulin (Ig) heavy and light chain-based scaffolding platform to bridge carrier and payload proteins. Payload systems for each strategy were produced in both *E. coli*- and plant-based expression systems and evaluated for efficacy with a plant-derived RTB-carrier.

In designing experiments to evaluate Strategy I, it was first necessary to investigate the nature of the RTA:RTB interaction in ricin toxin more closely. Identification of RTA structural domains involved in interactions with RTB can be found in the work of Montfort, et al. and Rutenber & Robertus dealing with the three-dimensional crystal structures of ricin and RTB, respectively.\(^1,2\) In addition to the single disulfide bond between Cys 259 of RTA and Cys 4 of RTB, there are nine polar and six hydrophobic interactions between RTA and RTB (see Figure 1a). The strategy is to identify RTA structural domains that are sufficient to mediate interaction and disulfide linkage to RTB. Fusion of these domains to the C-terminus of a payload protein may mimic the RTA:RTB interaction, where the active and toxic portions of RTA have been effectively replaced with a beneficial payload protein (see Figure 1b).
Strategy II, relied on the interaction between immunoglobulin (Ig) heavy- and light-chains (see Figure 2). Ig light chains (LC) are composed of two domains, termed variable ($V_L$) and constant ($C_L$). Heavy chains (HC) are composed of one variable ($V_H$) and three (or more) constant domains ($C_{H1} - C_{H3}$). Domains $V_H$ and $C_{H1}$ together are termed Fd. A single disulfide bond connects one HC to one LC at $C_{H1}$ and $C_L$. By fusing either LC or HC domains to RTB and the respective-interacting domain to payload proteins, I hoped to create a RTB-mediated “capture and carry” system.
Production of payloads in both *E. coli* and plants were investigated. Payloads produced in *E. coli* were purified and efficacy of interaction with a plant-derived RTB-carrier was assessed. One of the payload constructs used to test this strategy that was produced in *E. coli* contained a C-terminal tetracysteine (TC) motif, which has been shown to bind specifically to Lumio fluorescent reagents (Invitrogen, Carlsbad CA). Incorporation of the TC tag was thought to facilitate subsequent fluorescence microscopy of uptake in HT-29 cells. In the case of plant-derived payloads, the ability of plant cells to properly assemble separate HC and LC proteins to create a heterodimer was exploited. Rodriguez, et al. showed that when separate HC and LC gene cassettes (each gene is driven by separate promoter sequences) are placed on the same T-DNA, then transformed into tobacco by using an *Agrobacterium*-mediated system, full length antibodies were made. Hull, et al. has shown that individual strains of *Agrobacterium*, each harboring either HC or LC gene T-DNAs, can be mixed in equal volumes immediately prior to infiltration, and full-length, functional antibodies are produced. These studies prove that plant cells can synthesize correctly assembled Ig’s from separate, co-infiltrated HC and LC genes.
would like to take advantage of the plant’s ability to assemble these domains in our RTB carrier system, by genetic fusion of either a LC or HC domain to the RTB carrier and the corresponding HC or LC domain to the payload.

**Methods**

**PCR amplification of gene fragments for constructs**

Table 1 lists the various gene fragments used in these experiments, and primer sequences and templates used in PCR amplification reactions. PCR-generated fragments were gel purified (Qiagen, Valencia CA) and digested with the appropriate enzymes, then cleaned again before ligation. All constructs were first assembled in the pBC cloning vector (Stratagene La Jolla, CA). Following sequence confirmation, promoter:gene cassettes were subcloned into the pBIB-Kan$^5$ binary vector as HindIII/SalI or HindIII/Sacl fragments. The construct R6-2 has been described elsewhere$^6$, and served as the source for both the dual enhanced cauliflower mosaic virus 35S promoter/tomato etch virus (TEV) translational enhancer/patatin signal peptide fragment (de35S:TEV::sp) as well as the XhoI-GFP-Sacl fragment. All constructs used in plant-based expression incorporated the patatin signal peptide (sp) for targeting to the ER and secretion to the apoplast.$^7$
<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer sequences†</th>
<th>Template</th>
</tr>
</thead>
</table>
| *Nco*-Fd-*Sal* | 5'-GATATACCAGCTTCCAGCT (F)  
5'-CTCGAGTCGAGATACGAGTAACCAGGCAATTC (R) | pET-Fd * |
| *Nco*-Fd-stop-*Sal* | 5'-GATATACCAGCTTCCAGCT (F)  
5'-CTCGAGTCGACAACTGTGACAGATGCT (R) | pET-Fd * |
| *Sac*-AdIII*-stop*-XhoI | 5'-GAGCTCCTACCAGTTCAGTAATTAC (F)  
5'-CTCGAGTAATGGAACCTC (R) | Preprorinic b |
| *Xba*-AdIII:RTB-stop-*Sal* | 5'-GCTGATGTTTCTATGGATC (F)  
5'-CTCGAGTCAACAGATACGAGTAACCAGGCAATTC (R) | Preprorinic b |
| *Pst*-I-AdIII:RTB-stop-*Sal* | 5'-GTCCAGATCCTAGCGTAATTAC (F)  
5'-GTCGACATACGAGTAACCAGGCAATTC (R) | Mouse kappa LC * |
| *Xba*-C*-H*-1-stop-*Sal* | 5'-GCTGATGTTTCTATGGATC (F)  
5'-CTCGAGTAATGGAACCTC (R) | Mouse alpha HC * |
| *Xba*-C*-H*-1-XhoI | 5'-GTCCAGATCCTAGCGTAATTAC (F)  
5'-GTCGACATACGAGTAACCAGGCAATTC (R) | Mouse alpha HC * |
| *Xba*-Fd*-XhoI | 5'-GCTGATGTTTCTATGGATC (F)  
5'-CTCGAGTAATGGAACCTC (R) | Mouse alpha HC * |
| *Eco*-RI-Fd-stop-*Sal* | 5'-GATATACCAGCTTCCAGCT (F)  
5'-CTCGAGTCGAGATACGAGTAACCAGGCAATTC (R) | Mouse alpha HC * |
| *Eco*-RI-κLC-stop-*Sal* | 5'-GATATACCAGCTTCCAGCT (F)  
5'-CTCGAGTCGAGATACGAGTAACCAGGCAATTC (R) | Mouse alpha HC * |
| *Xba*-Fd*-XhoI | 5'-GCTGATGTTTCTATGGATC (F)  
5'-GATCCTCGAGAGAAAAAGGAGAAGGAGGGAGG (R) | Mouse alpha HC * |

† Underlined sequences represent restriction sites used in cloning and red sequences indicate stop codons incorporated into forward (F) and reverse (R) primers.

* pET-Fd was created by cloning a *Nde*I-Fd-*Sal* fragment into pET41a, not used here. The forward primer used to make this construct was 5'-GACTCATATG GTCCAGCTTCCAGTCT. The reverse primer used was the same used for *Nco*-Fd-*Sal* (see above).

b Preprorinic gene in pBI212, a gift of Dr. Sehnke.

c R6-2 is de35S:TEV::Pat:RTB-GFP in pHIB-kan, and is described elsewhere.

RTB-carrier constructs

Maps of the constructs screened as potential RTB-carrier conformations used in both Strategies I and II can be seen in Figure 3. The linker sequence used as a spacer between fusion partners was (Gly₃Ser)₃.
In creating constructs in which RTB comprised the C-terminal partner, the \textit{XbaI-RTB:linker-\textit{PstI}} fragment (which contains a \textit{XhoI} site between RTB and the linker) was first cloned into a modified pBC in which the \textit{XhoI} site in the polylinker had been eliminated (pBC-X) via Mung bean nuclease digestion and re-ligation, a gift of Dr. Maureen Dolan (ABI), to give the plasmid pBC-X:RTB:linker. Plasmid pBC-X:RTB:linker was then digested with \textit{XbaI} and \textit{XhoI} to release a \textit{XbaI-RTB-XhoI} fragment. Fragments such as \textit{XbaI-C}_{\text{L}}-\textit{XhoI}, \textit{XbaI-C}_{\text{H}}1-\textit{XhoI}, and \textit{XbaI-Fd-XhoI} could then be ligated into the vector fragment to yield pBC-X:C_{\text{L}}:linker, etc. The \textit{PstI-RTB-SalI} fragment was then ligated into these plasmids to yield fusions in which RTB was the C-terminal partner. In this way, all fusions between Ig domains and RTB contained the same linker sequence to maintain consistency. The GFP:AdIII:RTB construct includes both payload and carrier moieties, and is therefore included in Figure 3. In constructs A and B, AdIII refers to amino acids 182 through 267 of RTA.
Figure 3. Maps of plant-expressed RTB-carrier constructs evaluated in this study. All constructs were created in pBC or pBC-X cloning vectors. Promoter:gene cassettes were cloned into pBIB-Kan binary vector via HindIII/SalI or HindIII/SacI. Yellow boxes indicate the presence of the (Gly<sub>2</sub>Ser<sub>3</sub>)<sub>2</sub> linker peptide. Constructs A and B were used in RTA domain studies (Strategy I), constructs C through J were used in Ig scaffolding experiments (Strategy II). The position of restriction sites utilized in cloning and start and stop codons are indicated. These maps are not to scale.
Construction of pET41-TC *E. coli* expression vector

The pET41 (EMD Biosciences, San Diego CA) glutathione-S-transferase (GST) fusion vector for production of recombinant proteins in *E. coli* was modified to include the tetracysteine (TC) motif. The following oligonucleotides were annealed: 5’-CTGACTAAGCTTGAAGCTGGTGCTGTTGTCCTGGCTGTTGCAGGGTGCCGCCACCAGTGCTCGAGCTGACT (F) and 5’-AGTCAGCTCGAGACCGGTGCCGCCACCGCAACAGCCAGGACAACAAGCCACCAGCTTCAAGCTTAGTCAG (R). Underlined sequences indicate the TC motif (CCGPCC). The resulting fragment was digested with *Hind*III and *Xho*I and cloned into pET41b. The resulting plasmid, pET41-TC, contained the N-terminal GST tag, thrombin and enterokinase cleavage sites, a multiple cloning site for insertion of genes, the TC tag and a C-terminal 8HIS-tag (see Figure 4, Construct L).

*E.coli*-derived payload constructs

Maps of *E. coli*-produced payload constructs used in testing both RTA domain and HC:LC interaction strategies are shown in Figure 4. In construct K, AdIII refers to amino acids 178 – 267 of RTA.
Plant-produced payload constructs

Constructs created for testing the plant cell’s ability to assemble a HC:LC scaffolding between a co-expressed RTB-carrier and payload proteins (Strategy II) are depicted in Figure 5. In testing the plant cell’s ability to assemble carrier and payload in the RTA domain strategy, (Strategy I) constructs A and B were used (see Figure 3).

![Diagram of plant-derived payload constructs](image)

**Figure 5.** Maps of plant-derived payload constructs used in assessing feasibility of HC:LC scaffolding strategy (Strategy II). Both constructs N and O are pBIB-Kan-based plasmids and are driven by dual enhanced 35S CaMV promoter, and contain the TEV translational enhancer and patatin signal peptide (sp). In construct O, “*” denotes the presence of the endogenous HC hinge region between Fd and GFP.

*Agrobacterium tumefaciens*-mediated transient expression

pBIB-Kan plasmids harboring promoter:gene cassettes were transformed into *A. tumefaciens* strain LBA4404 using a modified freeze/thaw method. Positive clones were grown in 50 mL YEP medium containing 100 µg/mL kanamycin and 60 µg/mL streptomycin for 48 hr at 28°C, 220 rpm. To induce *A. tumefaciens* prior to infiltration, cell pellets were harvested via centrifugation (5000 X g for 10 min), resuspended in 300 mL induction media (20 mM MES pH 5.5, 0.3 g/L MgSO₄ · 7H₂O, 0.15 g/L KCl, 0.01 g/L CaCl₂, 0.0025 g/L FeSO₄ · 7H₂O, 2 mL/L 1 M NaH₂PO₄ pH 7.0, 10 g/L glucose) containing 100 µg/mL kanamycin and 60 µg/mL streptomycin, supplemented with 0.2 µM acetosyringone and incubated at 28°C, 220 rpm, for 4
hr to overnight. Induced *A. tumefaciens* cultures were introduced into four to six week old *Nicotiana benthamiana* plants either by pressure injection or vacuum infiltration. For pressure injection, a disposable syringe without a needle was filled with *A. tumefaciens* culture and pressed against the underside of the leaf.\(^{12}\) For vacuum infiltration, plants were place upside-down in a beaker containing the induced culture so that all aerial portions were submerged. This was then placed inside a vacuum chamber and vacuum was applied (approximately 1 min) and broken by abruptly pulling off the tube from the chamber.\(^{13}\) This procedure was performed twice for each plant to ensure complete infiltration. Following infiltration, plants were returned to their growth chambers and allowed to incubate for 48 – 72 hr.

**Enrichment of RTB-containing fusion proteins using immobilized lactose**

For initial characterization of RTB-containing transgenes products, infiltrated leaves were ground to a fine powder under liquid nitrogen (LN\(_2\)) using a mortar and pestle. Extraction buffer 1 (100 mM Tris, 100 mM Ascorbic acid, 150 mM NaCl, 20 mM EDTA, 2.5% PVPP) was added to the powder in a 2:1 volume buffer:mass leaf ratio. This crude extract was then centrifuged at 14,200 X g for 30 min at 4°C. The supernatant was filtered through KimWipes to yield the cleared extract. Cleared extracts were batched with immobilized lactose resin (EY Laboratories, San Mateo CA) for 10 – 30 min at RT. Resin was collected by pouring the mixture into an empty disposable chromatography column and washed with 10 column volumes of PBS. RTB-containing fusion proteins were eluted by washing with 3X 1 column volumes of 0.5 M D-galactose in PBS.
Extraction and purification of RTB-containing fusion proteins

For experiments that required greater levels of purity, such as uptake studies, 10 – 20 g of infiltrated leaves were ground under LN₂ in a mortar and pestle to a fine powder. 50 mL of extraction buffer 2 (100 mM Tris-HCl pH 7.5, 20 mM D-galactose, 1% PVPP) was added to the powder and allowed to thaw at RT. The resulting crude extract was centrifuged at 14,200 X g for 30 min at 4°C. The supernatant was filtered through KimWipes, brought to 100 mL with distilled H₂O and the pH was adjusted to 7.5 with 1 N NaOH. This cleared extract was then filtered though a 0.45 µm membrane and loaded onto an equilibrated 20 mL column volume MacroPrep High Q (Bio-Rad, Hercules CA) column using a Bio-Rad Duo-Flow FPLC system. Following loading of the sample, the column was washed with 80 mL of 50 mM Tris-HCl pH 7.5. The RTB-containing proteins were eluted and collected from the column by washing with 45 mL 50 mM Tris-HCl pH 7.5, 400 mM NaCl. The column was then cleaned by washing with 50 mM Tris-HCl pH 7.5, 1 M NaCl and re-equilibrated with 50 mM Tris-HCl pH 7.5. The RTB-containing sample (400 mM NaCl) was loaded onto a 1 mL immobilized lactose column (EY Laboratories, San Mateo CA) and washed with PBS. Purified RTB and RTB-containing fusion proteins were eluted by washing with 4 X 1 mL PBS + 500 mM D-galactose. RTB-containing samples were then concentrated using YM-10 Centricons (Millipore Corp., Bedford MA) and dialyzed to PBS. Concentrated, dialyzed samples were then analyzed via Western blot using anti-RTB antibodies (see Chapter 2), silver stained SDS-PAGE, and asialofetuin binding assay.

Extraction and purification of E.coli-produced payload proteins

E. coli strain BL21(DE3) harboring Construct K (GST:AdIII) was grown in a 5 mL overnight LB culture containing kanamycin (100 µg/mL). This 5 mL culture was used to induce
a 1 L LB (+ 100 µg/mL kanamycin) culture. The 1 L culture was grown at 37°C at 225 rpm for ~ 3 hr or until the OD$_{600}$ reached ~ 0.6. The culture was induced by the addition of isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After 3 hr of induction at 37°C, 225 rpm, the cell pellet was recovered by centrifugation and resuspended in 30 mL cell lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 0.1% Triton X-100, 5 mM MgSO$_4$, 1 mg/mL lysozyme, 10 µg/mL DNase I and 10 µg/mL RNase A) and quickly frozen in LN$_2$, then allowed to thaw at RT. Lysed extract was centrifuged for 30 min at 14,200 X g at 4°C, and inclusion bodies were recovered. No detectable fusion protein was observed in the soluble fraction, based on immobilized reduced glutathione (GST-Bind) chromatography. Insoluble GST:AdIII was refolded by first dissolving the inclusion bodies in 6 M urea, 50 mM Tris HCl, pH 8.0, 5 mM DTT and then removing the urea through successive rounds of dialysis. Dialysis buffers for removal of urea omitted DTT but contained 2.5 mM cysteine and 0.5 mM cystine, a redox pair used in facilitating disulfide bond formation. Following two additional rounds of dialysis against 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM DTT, the sample was applied to 2 mL of GST-Bind resin (EMD Biosciences, San Diego CA). The resin was then washed with 20 mL 50 mM Tris HCl pH 8.0, 150 mM NaCl, 5 mM DTT, and bound GST:AdIII was eluted by washing 3X 2 mL 50 mM Tris HCl pH 8.0, 25 mM reduced glutathione (glut$_{red}$).

For recovery of GST:Fd:TC and GST:Fd, cultures of E. coli strains BL21(DE3) harboring either Construct L (GST:Fd:TC) or Construct M (GST:Fd) were inoculated in the same way described above. After 3 hr of growth at 37°C, 225 rpm (or when the OD$_{600}$ ~ 0.6), the cultures were cooled by incubating on ice 5 – 10 min, then IPTG was added to a final concentration of 0.5 mM. Cultures were induced for 4 hr at 25°C, 225 rpm, then cell pellets were harvested by centrifugation. The cells were lysed by resuspension in 30 mL lysis buffer
and application of a freeze/thaw cycle (described above). Lysed extracts were cleared via centrifugation at 14,200 X g, for 30 min at 4°C. Cleared extracts were applied over a 2 mL GST-Bind resin column, and the resin was washed with 20 mL 50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM DTT. GST:Fd:TC or GST:Fd was eluted from the column by washing with 3X 2 mL 50 mM Tris HCl pH 7.5, 25 mM glutared. For cleavage of the purification tag using thrombin, washed GST-Bind resin containing bound protein was suspended in 2 mL 50 mM Tris HCl pH 7.5, 150 mM NaCl, 20 mM CaCl$_2$, 5 mM DTT, and 1 unit of thrombin protease was added. The reaction occurred at RT for 4 hr, and cleaved protein was recovered by collecting the liquid fraction of the mixture (the GST tag remained bound to the resin) and washing the resin with 50 mM Tris HCl pH 7.5, 150 mM NaCl, 5 mM DTT. Fractions were pooled and concentrated.

**Asialofetuin binding assay.**

A functional ELISA utilizing asialofetuin instead of a capture antibody was employed to assess galactose-specific lectin activity and quantify rRTB and RTB-containing fusion proteins. Asialofetuin is a modified mammalian glycoprotein that contains galactose-terminated glycans (Sigma, St. Louis MO). Asialofetuin at 300 µg/mL in PBS was bound to the wells of an Immulon 4HBX plate for 1 hr at RT. The wells were then blocked with 3% BSA in PBS for 1 hr at RT. Castor bean-derived RTB (cbRTB; Vector Labs, Burlingame CA) was used for the standard curve, ranging from 1.95 to 250 ng/well in PBS + 10 mM D-galactose. For asialofetuin binding, 100 µL of standards and samples were incubated at RT for 1 hr. The plate was then washed 3X with PBS (300 µL/well). Rabbit anti- *Ricinus communis* lectin antibody (Sigma R-1254), diluted to 1:4000 in blocking buffer was then added (200 µL/well) and allowed to incubate for 1 hr at RT. Wells were washed again and alkaline phosphatase-labeled goat anti-
rabbit antibody (Bio-Rad, Hercules CA), diluted 1:4000 in blocking buffer, was added and allowed to incubate for 45 min at RT. The wells were washed a third time and alkaline phosphatase substrate (100 µL/well; Pierce, Rockford IL) was applied. After the color developed sufficiently (10 – 15 min), the reaction was stopped with the addition of 50 µL 2 N NaOH and the absorbance at 405 nm was read. The inverse of the absorbance at 405 nm was plotted vs. the inverse of the standard RTB/well to give a linear relationship. This equation was then used to estimate the amount of RTB in the samples in terms of RTB equivalents.

By probing with antibodies other than the rabbit anti-\textit{Ricinus communis} lectin antibody used for quantification, it was possible to use this assay to determine carrier/payload interactions. For example, in experiments in which GFP served as the model payload, binding of RTB-carrier to the payload was assessed by applying the samples to asialofetuin-coated wells and probing with anti-GFP antibodies. By inclusion of the proper controls, a positive reaction in this scenario indicated that GFP must be associated with RTB in order to bind to asialofetuin. Probing the same sample with different antibodies (in different wells) allowed determination of what proteins were present in the sample that had lectin-positive activity.

Cell uptake and fluorescence microscopy

See Chapter 2, Methods.
Results

Section 1: Development of RTB-carrier proteins

Strategy I: Carriers used to evaluate RTA domain-mediated interaction with payload

Recombinant RTB (rRTB, product of Construct C) and castor bean-derived RTB (cbRTB) were used as the carrier portion of the system to study the possibility of utilizing an *E. coli*-produced payload. The aim was to determine if AdIII could mediate interaction with un-modified RTB. Results of these experiments are discussed below. The expression, purification, and characterization of rRTB is discussed in detail in Chapter 2.

Strategy II: Evaluation of potential carrier constructs in engineering an Ig domain scaffolding between carrier and payload

The potential of using the strong interactions between immunoglobulin (Ig) light and heavy chains as part of an RTB capture and carry system (Strategy II) was also tested. To develop the RTB-carrier component for Strategy II, a suite of fusion constructs between various Ig HC and LC domains and RTB were constructed, incorporating many possible orientations with regard to N- and C-terminal partner (Constructs D through J, Figure 3). The Ig domains used were \( C_L \) (LC constant region), the entire mouse kappa light chain (\( \kappa LC \)), \( C_{H1} \) (HC constant region 1), and the Fd portion of the mouse alpha heavy chain. All of these domains contribute to the disulfide bond between HC and LC in full-length Ig’s. Table 2 lists the constructs tested, their ability to drive product accumulation in *Agrobacterium*-infiltrated leaves, and the quality of product.
Table 2: Evaluation of potential RTB-carrier constructs using Strategy II

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fusion</th>
<th>Product recovery</th>
<th>Quality of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>Fd:RTB</td>
<td>++</td>
<td>Breakdown</td>
</tr>
<tr>
<td>E</td>
<td>RTB:Fd</td>
<td>-</td>
<td>n/a</td>
</tr>
<tr>
<td>F</td>
<td>RTB:κLC</td>
<td>+</td>
<td>Intact</td>
</tr>
<tr>
<td>G</td>
<td>RTB:C_h1</td>
<td>++</td>
<td>Breakdown</td>
</tr>
<tr>
<td>H</td>
<td>RTB:C_L</td>
<td>++</td>
<td>Breakdown</td>
</tr>
<tr>
<td>I</td>
<td>C_h1:RTB</td>
<td>+++</td>
<td>Breakdown</td>
</tr>
<tr>
<td>J</td>
<td>C_h1:RTB</td>
<td>++</td>
<td>Breakdown</td>
</tr>
</tbody>
</table>

a Relative expression levels were assessed via asialofetuin binding assay.
b Quality of product refers to relative amount of full-length fusion protein compared to breakdown product as visualized on anti-RTB western blots (see Figure 7); n/a: not applicable

Constructs were assessed in regards to amount of full-length fusion protein (using predicted molecular weight estimates) via western immunoblot analysis using anti-RTB specific antibodies. Figure 6 shows representative blots from these experiments. Constructs in which the fusion partner was on the N-terminus of RTB all showed a similar pattern of breakdown, with the major proportion of lactose-enriched protein migrating to the same position on SDS-PAGE as cbRTB and rRTB. Characterization of this breakdown and evidence for the presence of proteolytic-susceptible sites within RTB are presented in Chapter 4 and will be discussed in much more detail there.

Of the seven constructs tested, only Construct F (RTB:κLC) generated a product that bound to lactose/asialofetuin, reacted with anti-RTB antibodies, and the majority of product matched the predicted molecular weight (~55 kD).
kD) corresponding to the full-length fusion (Figure 6, lane 8). However, RTB:κLC also had the lowest expression level among constructs which generated a product. Due to the observation that the vast majority of lectin-positive fusion was full length, RTB:κLC was chosen to move forward for payload capture studies.

Development of RTB-specific purification protocol

In order to achieve a higher level of purity of plant-produced RTB-carrier fusion proteins, a more rigorous purification regime was optimized. One-step chromatography using immobilized lactose enriched for proteins consisting of or containing a functional RTB unit, whether in the form of a full-length fusion or breakdown product, but many endogenous plant proteins co-purified. Many different chromatography supports were tested as a possible first step preceding lactose affinity, including cation exchange (both strong “S” and weak “CM”), anion exchange (strong “Q” and weak “DEAE”), hydroxyapatite, and hydrophobic interaction chromatography (following ammonium sulfate precipitation), to varying degrees of success (data not shown). Among these procedures, anion exchange resins appeared most effective in selectively enriching RTB and RTB-fusions. Utilizing the MacroPrep High Q resin (Bio-Rad, Hercules CA), I was able to determine precise conditions in which RTB and RTB-fusions bound and eluted.

The purification strategy used for purification of rRTB and all other RTB-fusions created thus far in our lab, in both the current research and others not discussed here, was developed using leaves expressing Construct I, C_L:RTB. This particular fusion was chosen for several reasons. First, the expression of asialofetuin-binding, anti-RTB-reactive proteins was relatively high with this construct, as determined by asialofetuin binding assays. Secondly, the presence of
full-length product and two distinguishable breakdown bands allowed for developing conditions under which any RTB-containing protein could be purified using the same extraction buffer and elution conditions. The exact same protocol is used to purify a number of different RTB-containing fusion proteins, including RTB:κLC, RTB:GFP, rRTB (See Chapter 2), and IL12:RTB (See Liu & Cramer, 2006). All fusion partners tested to date purified using this protocol, indicating that the properties of RTB (overall charge, etc.) are being exploited, and not those of the fusion partner. Figure 7 shows a silver stained SDS-PAGE gel of purified proteins. Note the difference in purity between C\textsubscript{L}:RTB that has been enriched via lactose affinity alone (lane 1) and C\textsubscript{L}:RTB that has undergone purification through anion exchange followed by lactose affinity (lane 2). The three bands seen in lane 2 all contain RTB, as determined through anti-RTB western blots (see Figure 6, lane 4) and N-terminal sequencing of the two ~30 kD bands (See Chapter 4). The high molecular weight band in lane 2 corresponds to full-length C\textsubscript{L}:RTB (predicted size 42 kD). The one band at ~55 kD in lane 3 is RTB:κLC. When leaves infiltrated with empty-vector A. tumefaciens are subjected to this purification regime, little to no proteins are visualized via silver stain (lane 4). This indicates that this purification regime is specific to RTB and RTB-containing fusion proteins.
**Strategy II: Characterization of RTB:κLC carrier**

In order to assess whether RTB:κLC is of the proper conformation necessary for association with Fd, its natural partner (Fd), SDS-PAGE analysis under non-reducing and reducing conditions was performed. RTB:κLC exhibits slightly different migration patterns on SDS-PAGE under reducing and non-reducing conditions. This difference in migration can be seen in Figure 8. Non-reduced RTB:κLC runs slightly faster than reduced RTB:κLC, probably due to the presence of unbroken internal disulfide bonds that condense the structure of the fusion. There are four internal disulfide bonds in RTB and two in κLC. Reducing these bonds allows for an unrestrained, more open structure that exhibits a higher apparent molecular weight than non-reduced RTB:κLC. These data suggest that the plant cell is properly folding RTB:κLC.

**Section 2: Evaluation of assembly between E. coli-derived payloads and RTB-carrier**

**Strategy I: Refolding of E. coli-produced payload and evaluation of interaction with RTB carrier**

In order to test whether the C-terminal domain of RTA (AdIII) was sufficient to mediate interaction with RTB analogous to RTA:RTB association observed in ricin toxin, AdIII was fused to the C-terminus of glutathione-S-transferase (GST) by cloning the appropriate DNA fragment into pET41a (Construct K; see Figure 4). Production of this fusion was problematic in that no significant levels of soluble protein were produced under a battery of different induction conditions.
conditions (decreased induction temperatures and incubation times, lower IPTG concentrations, etc.). Therefore insoluble material was refolded by first dissolving the inclusion bodies in a buffer containing 6 M urea and through successive rounds of dialysis in the presence of the cysteine/cystine pair, the urea was removed by half each round. Following the complete removal of urea, the sample was dialyzed to conditions optimal for binding to immobilized reduced glutathione (glut\text{red}). A large portion of refolded material bound and eluted from the GST-Bind resin, indicating successful refolding of the GST portion of the fusion protein. Coomassie-stained GST-Bind chromatography samples can be seen in Figure 9a.

To assess the ability of GST:AdIII to interact with RTB, two tests were performed. Refolded GST:AdIII was mixed with cbRTB in a 1:1 molar ratio in the presence of 1 mM DTT and dialyzed to PBS 2X. In the first test, the dialyzed mixture was applied to a modified asialofetuin assay that utilized anti-GST antibodies as the detection antibody. Successful interactions would be indicated by a positive reaction to the probe antibody. GST:AdIII bound directly to the plate (that is, no asialofetuin), was used as a control and reacted with the antibody. The samples applied to

![Figure 9. Refolding of Strategy I E. coli-produced payload (GST:AdIII) and evaluation of interaction with RTB. A: Refolding of insoluble GST:AdIII from inclusion bodies. Insoluble protein was dissolved in 6 M urea buffer and refolded by successive rounds of dialysis in which the urea was removed by ½ each round, in the presence of the cysteine/cystine redox pair (see Methods). Success of refolding was assessed by binding to and elution from GST-Bind resin. Lane 1: Refolded GST:AdIII. Lane 2: Flow-through of GST-Bind (the portion of refolded protein that did not regain activity). Lane 3: wash fraction of GST-Bind chromatography. Lane 4: Eluted GST:AdIII (the portion of refolded protein which did regain activity). The predicted molecular weight of GST:AdIII is 44 kD. B: Assessment of GST:AdIII interactions with RTB. Refolded GST:AdIII was mixed with RTB (1:1 molar ratio) in the presence of reductant (1 mM DTT) and dialyzed to PBS 2X. Binding was evaluated via non-reduced SDS-PAGE; a shift to higher molecular weight positions in non-reduced samples (denoted by green dot) would indicate successful interactions. Lane 1: GST:AdIII + RTB reduced. Lane 2: GST:AdIII reduced. Lane 3: GST:AdIII + RTB non-reduced. Lane 4: GST:AdIII non-reduced.](image-url)
asialofetuin-coated wells did not react with anti-GST antibodies, but did react to anti-ricin antibodies (data not shown), indicating that binding between GST:AdIII and cbRTB did not occur. The second test involved inspection of migration patterns on SDS-PAGE under reducing and non-reducing conditions. A higher molecular weight band, at approximately the sum of the molecular weights of GST:AdIII (44 kD) and cbRTB (32 kD) should be present in non-reduced lanes if interactions did occur. As seen in Figure 9b, lane 3, assembled products of this size was not observed. Thus, in contrast to E. coli-synthesized RTA, domain III alone does not appear effective in mediating in vitro assembly with RTB.

**Strategy II: Expression and purification of E.coli-derived payloads and evaluation of interaction with RTB:κLC**

Since the selected Strategy II carrier is RTB:κLC, the corresponding payload must contain Fd, the portion of the HC that is composed of V\text{H} and C\text{H}1, the domains that interact with LC in immunoglobulins (see Figure 2). The aim of these experiments was to determine if a Fd-containing payload protein produced in E. coli could bind to RTB:κLC produced in the plant expression system. Constructs L and M (see Figure 4) were expressed and the product was purified as described above. Construct L (GST:Fd:TC) contained the tetracysteine (TC) tag, a motif that preferentially binds a biarsenical fluorescent reagent, such as 4’,5’-bis(1,3,2-dithioarsolan-2-yl) fluorescein, marketed as Lumio Green® (Invitrogen, Carlsbad CA). This motif was included into the payload to facilitate downstream fluorescent microscopy of uptake experiments. GST:Fd:TC was expressed in a soluble form, and approximately 1.5 mg of purified protein was recovered per liter of culture, as determined by Bradford analysis of purified samples. To ensure that the GST tag did not interfere with binding to RTB:κLC, thrombin
protease (EMD Biosciences, San Diego CA) was added to GST:Fd:TC bound to immobilized glut_red resin (GST-Bind). Digesting the protein with protease while immobilized allowed for complete removal of the tag, by freeing Fd:TC from the resin which is collected by washing with buffer. Subsequent elution of the resin-bound material revealed that virtually no intact GST:Fd:TC remained, as only a band matching the predicting molecular weight of GST was observed via Coomassie-stained SDS-PAGE (see Figure 11).

Addition of the Lumio Green ™ reagent to samples prior to gel-loading allowed for evaluation of the TC tag function. The gel was photographed on a standard UV light box set-up after running and prior to staining. Only proteins containing the TC tag and therefore the Lumio Green ™ reagent fluoresced when exposed to UV light (see Figure 10). This type of analysis proved useful in expression and purification optimization studies (data not shown).

To evaluate whether E.coli-produced Fd:TC could interact with plant-derived RTB:κLC, both proteins were purified, mixed in 1:1 molar ratios and dialyzed to PBS. Binding was assessed via a modified asialofetuin assay that used goat anti-mouse alpha chain probe antibody (Sigma A-4937). Fd:TC bound directly to the wells reacted well with this antibody, but no binding between RTB:κLC and Fd:TC was observed. Uptake studies using HT-29 cells also showed no binding (data not shown).
shown), both when the Lumio Green ® reagent was added to the sample before applying to the cells, and when the Lumio Green ® reagent was added to cells that had undergone incubation with the sample.

To determine if the TC or poly-histidine tags on the C-terminus of Fd:TC prevented binding to RTB:κLC, Construct M (GST:Fd) was developed, which included a stop codon at the 3’ end of the Fd gene. The expression and purification of GST:Fd was identical to that of Fd:TC. GST:Fd and Fd were labeled with NHS-fluorescein (see Chapter 2) to facilitate fluorescence microscopy. No assembly between Fd and RTB:κLC was observed using the same tests as described above. Thus it appears that the immunoglobulin Fab domain can not be efficiently reconstituted in vitro using components independently produced in plants (RTB:κLC) and E. coli (heavy chain Fd and derivatives).

**Section 3: Evaluation of plant-produced payload interactions with RTB-carrier when co-expressed**

The results described above suggest that the efficiency of in vitro assembly of components individually synthesized in plants (carrier) and bacteria (payload) is not sufficient to support capture and carry platforms based on either Strategy I (RTA-domain interactions with RTB) or Strategy II (Ig domain scaffolding). I therefore designed strategies to assess the efficacy of these interactions under conditions where both components were co-synthesized in plant cells.
Strategy I: RTA-domain mediated interactions between payload and carrier

In the case of plant-produced payload proteins in Strategy I, Construct B (GFP:AdIII:RTB) was employed. Construct B contains both carrier and payload in the same construct, and is therefore discussed here. The rationale behind Construct B was to replace the DNA encoding amino acids 1 – 181 of RTA with the gene encoding green fluorescent protein (GFP), as arranged in the native preproricin gene. The construct included the “proricin” 12 amino acid vacuolar-targeting linker. The idea was to see if the gene product of Construct B could mimic native ricin toxin processing, with the end result being GFP:AdIII bound to RTB via a disulfide bond between RTA Cys 259 (of AdIII) and RTB Cys 4 (see Figure 1 for a theoretical representation). In these experiments, Construct A (AdIII:RTB) served as a control.

Constructs A and B were expressed using the Agrobacterium-mediated transient expression system. After 48 hr of incubation, RTB-containing proteins were enriched from leaf material using immobilized lactose chromatography. Elution fractions were separated on non-reducing 10% Tris-Glycine SDS-PAGE gels (Invitrogen) and transferred to nitrocellulose for western blot using anti-RTB or anti-GFP (CloneTech) specific antibodies.

A ~ 60 kD band that reacts with both anti-RTB and anti-GFP should be

![Image of western blot analysis](image.png)
present from Construct B in order for this strategy to be successful. As seen in Figure 11, no such band is present, even after a 3 hr exposure. In addition, very little ~ 30 kD band representing RTB is present (seen in only the 3 hr exposure frame). In contrast, I typically observe a strong cross-reacting band with only 1 – 5 min exposure using constructs containing RTB:GFP in this system (see Figure 12, lane 2). These results indicate that RTA domain III alone is insufficient to mediate interaction between GFP:AdIII and RTB. Furthermore, the absence of a strong band from Construct A indicates that these constructs may produce an unstable gene product.

Strategy II: Assembly of co-expressed RTB:κLC carrier and Fd:GFP payload by plant cell

Since the carrier using Strategy II is RTB:κLC, the corresponding payload must contain the Fd portion of HC. Constructs consisting of Fd or Fd plus the HC hinge region (Fd*; see Figure 2) fused to the N-terminus of GFP were prepared (Constructs N and O, see Figure 5). In order to demonstrate effective and specific assembly of the RTB:κLC carrier with Fd-containing payloads, a variety of constructs were transiently expressed in leaves alone or in combination (see Table 3).

<table>
<thead>
<tr>
<th>Treatment ID</th>
<th>Constructs Infiltrated</th>
<th>Gene Products</th>
<th>Individual expected size</th>
<th>Expected size of assembled products</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>pBIB-Kan (empty vector)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>II</td>
<td>R6-2</td>
<td>RTB:GFP</td>
<td>60 kD</td>
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</tr>
<tr>
<td>III</td>
<td>F &amp; N</td>
<td>RTB:κLC &amp; Fd:GFP</td>
<td>55 kD &amp; 51 kD</td>
<td>106 kD</td>
</tr>
<tr>
<td>IV</td>
<td>F &amp; O</td>
<td>RTB:κLC &amp; Fd*:GFP</td>
<td>55 kD &amp; 52 kD</td>
<td>107 kD</td>
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<tr>
<td>V</td>
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</tr>
<tr>
<td>VI</td>
<td>C &amp; O</td>
<td>rRTB &amp; Fd*:GFP</td>
<td>32 kD &amp; 52 kD</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*see Medina-Bolivar, et al.*

b Two constructs were simultaneously infiltrated into the same plant by mixing equal volumes of induced A. tumefaciens cultures immediately prior to infiltration.
Crude leaf extracts generated from the treatments listed in Table 3 were tested for the presence of interactions between RTB and GFP. Figure 12 shows the results of these assays. All treatments except empty-vector control (treatment I) gave positive responses when bound to asialofetuin and probed with anti-ricin antibodies, indicating the presence of RTB-containing proteins in the crude extracts of treatments II through VI. A standard sandwich GFP ELISA was also performed, in which wells were coated with monoclonal anti-GFP specific antibodies and probed with polyclonal (rabbit) anti-GFP. GFP ELISA data confirmed the expression of GFP-containing constructs by the plant in treatments II through VI. Demonstration of a κLC:Fd scaffold bridging RTB to GFP was accomplished by comparing the responses of asialofetuin-bound samples probed with both anti-ricin and anti-GFP specific antibodies (both developed in rabbit), in separate wells (see Figure 12). Only RTB:GFP (treatment II) and RTB:κLC co-expressed with either Fd:GFP (treatment III) or Fd*:GFP (treatment IV) gave positive reactions when asialofetuin-bound samples were probed with anti-GFP antibodies. These data indicate that the assembly of RTB-carrier and GFP-payload by the plant cell is mediated by κLC:Fd interactions.

In order to assess the ability of κLC:Fd interactions to mediate co-purification of carrier and payload, selected treatments were subjected to the standard RTB-specific purification
protocol described above and analyzed via anti-RTB western blots. As shown in Figure 13, lanes 4 and 5, RTB-purified samples from leaves co-expressing RTB:κLC with either Fd:GFP (treatment III) or Fd*:GFP (treatment IV) showed bands of the expected sizes using both anti-RTB and anti-GFP detection antibodies. In contrast, no GFP was recovered from purified fractions from leaves co-expressing rRTB and Fd:GFP (Figure 13, lane 3). The higher molecular weight band seen in Figure 13, lanes 4 and 5 of both immunoblots is consistent with the size expected of the expected dimer (~ 110 kD) and suggest that the samples may not have been fully reduced. RTB:GFP (treatment II) served as a positive control in these experiments. These data show that Fd:GFP co-purifies with RTB:κLC, but not rRTB, using a RTB-specific purification regime. These findings are consistent with κLC:Fd scaffolding bridging RTB-carrier and GFP-payload.

This two component system based on Ig domain scaffolding was designed to mimic RTA:RTB interactions, which involve a single intermolecular sulphydryl bridge that is reduced in the ER of mammalian target cells permitting dissociation. In order to test whether assembled product from RTB:κLC and Fd:GFP co-expressed in plant cells was sulphydryl-linked, I compared electrophoretic mobility in the presence and absence of reducing agent (DTT).
Purified protein from treatment IV was run on 10% SDS-PAGE under both reducing and non-reducing conditions and both silver stained and transferred to nitrocellulose for anti-RTB western analysis. As shown in Figure 14, RTB:κLC and Fd*:GFP in the presence of reductant migrate to their respective molecular weights. Under non-reducing conditions, the proteins migrate much slower, corresponding to a RTB:κLC and Fd*:GFP heterodimer (lanes 4). These data indicate that RTB:κLC and Fd*:GFP are held together via disulfide bonding.

**Assessment of uptake function of plant-produced Strategy II carrier/payload system**

Mammalian cell uptake studies were used to assess the ability of the RTB:κLC carrier to successfully deliver its model payload Fd:GFP, in addition to mediating co-purification of co-expressed proteins, in order to show that κLC:Fd serves as a scaffolding to link a payload protein to RTB-carrier. Purified proteins from treatments II, III, IV and V (see Table 3) were applied to HT-29 cells as described in Chapter 2 and uptake was assessed by fluorescence microscopy at 30, 60, and 120 min after binding (see Methods). rRTB labeled with NHS-fluorescein (rRTB-fl) and RTB:GFP served as positive controls (see Chapter 2). As seen in Figure 15, fluorescence microscopy using a GFP filter showed that RTB:κLC, but not rRTB, mediated uptake of Fd:GFP. This is consistent with the analyses described above which showed that Fd:GFP co-purifies with RTB:κLC but not rRTB when co-expressed in plant leaves. At T = 0, cell surface-bound GFP is clearly visible in the positive controls and protein from treatments III and IV. By
T = 60, clear punctate structures are observed which increase by T = 120, indicating internalization and accumulation in endosomal and/or lysosomal compartments. No fluorescence was observed at any time point in rRTB + Fd:GFP samples. GFP alone (Clontech) applied to cells as a negative control likewise showed no binding (Figure 15 lower insert) or uptake into HT-29 cells.

<table>
<thead>
<tr>
<th></th>
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<th>T = 30</th>
<th>T = 60</th>
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<td>GFP T = 0</td>
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Figure 15. Fluorescence microscopy of Fd:GFP delivery into cells mediated by RTB:κLC. Purified proteins from the treatments listed in Table 3 were applied to HT-29 cells, incubated at 4°C for 30 min to allow binding to cell surface, then washed with cold HBSS. Photographs of fluorescence using GFP filter set were then taken: T = 0. Cells were then incubated at 37°C and additional photographs were taken at specified time points (min) to monitor the uptake in real time. rRTB-fl is fluorescein-labeled recombinant RTB (see Ch. 2), which along with the direct fusion RTB:GFP serves as positive controls. At left is cells treated with GFP alone.
Discussion

I have developed a modular, “plug and play” RTB-mediated protein delivery system. Of the two strategies tested, exploitation of RTA-domains to mediate interaction with RTB (Strategy I) and utilization of an Ig domain scaffold to bridge carrier and payload (Strategy II), only Strategy II implemented in a plant-based production system produced the flexibility and efficiency desired for this technology. By taking advantage of the plant cell’s ability to assemble co-expressed κLC- and Fd-containing fusion proteins, I have shown that RTB:κLC can capture payload proteins (in this case Fd:GFP) co-expressed in the same cell, facilitate purification using a RTB-specific protocol, and mediate uptake in human epithelial cells. Additionally, I have shown that the interaction between carrier and payload includes a disulfide bond, which may allow for dissociation of carrier and payload once internalized by the target cell.

Plants are well suited for production of a modular system of this type. The Agrobacterium-mediated transient expression allows for great flexibility in terms of the number of different genes which can be co-expressed in a single plant, by simply mixing different Agrobacterium strains immediately prior to infiltration.

Payload proteins produced in E. coli for test strategies did not bind to their respective RTB-carriers. In the case of Strategy I, this may be due to the fact that the payload underwent a refolding process that, while restoring activity to the GST portion of the fusion, may not have been efficient in producing a properly refolded AdIII moiety. However, subsequent to the initiation of this strategy, a report was published\(^\text{15}\) indicating the lack of RTA His 40 is more likely to be responsible for the failure of GST:AdIII to associate with RTB. Of the sixteen interactions between RTA and RTB mentioned earlier, only His 40 and Glu 41 do not reside within the last 85 amino acids of RTA. His 40 and Glu 41 of RTA form the bend point for a
loop that interacts with Asp 94 and Lys 219 of RTB, respectively. His 40 of RTA and Asp 94 of RTB, in addition to this interaction, also make up two of the three residues that form a recently discovered lipase active site (the third residue is Ser 221 of RTA). When researching this lipase activity, Morlon-Guyot, et al. discovered that when His 40 of RTA was mutated to Ala, the resulting protein associated only poorly with RTB, confirming the importance of this interaction.\textsuperscript{15} This idea is bolstered by the failure of the GFP:AdIII:RTB construct, produced in plants, to mimic the natural processing of preproricin. The \textit{E. coli}-produced proteins for use in evaluation Strategy II, GST:Fd:TC, Fd:TC, GST:Fd, and Fd did not assemble with RTB:κLC. This observation reinforces the notion that assembly of κLC and Fd into a heterodimer relies on co-expression of κLC and Fd genes and possibly chaperone-mediated assembly within the same cell, as immunoglobulins are naturally produced. I am not aware of any reports in which antibodies or Fab fragments have be assembled from independently synthesized and purified HC and LC proteins.

Linking a payload to the RTB-carrier by using this Ig scaffolding has benefits for certain applications over chemical conjugation and direct genetic fusion. Allowing the plant cell to assemble separate, co-expressed carrier and payload molecules precludes the need to perform unpredictable and possible inefficient chemical conjugation chemistries. Direct genetic fusion of payload to RTB produces numerous concerns. First, the stability of different RTB-payload fusions varies greatly, as seen in Table 2. Secondly, direct fusion, without incorporation of a proteolytic recognition site, is generally unbreakable and therefore the payload is obliged to traffic to which ever compartment RTB goes to when endocytosed. For example, vaccine antigens designed for presentation via the MHC class I pathway may remained trapped within the endosomal and/or only presented via MHC class II pathways (see Chapter 2, Figure 1).
disulfide bond may be broken through changes in reduction potential and/or enzymatic activity, and perhaps by including sub-cellular localization signals on the payload it may be possible to direct carrier and payload to different compartments upon internalization.

Possible applications of this system include vaccines and enzyme replacement therapy (ERT). Several groups, including ours, are currently using RTB or ricin to facilitate immune response to antigen proteins, as described earlier. ERT, especially in the area of lysosomal storage disorders, is an attractive candidate application because of the characteristics of RTB trafficking upon endocytosis in target cells. Only a small fraction of internalized RTB/ricin moves through the retrograde ER pathway, while the majority moves to endosomal and lysosomal compartments. Exploiting this natural tendency of RTB may prove very effective in delivery of lysosomal proteins such as glucocerebrosidase and iduronidase. Current ERT strategies rely on in vitro manipulation to provide the presence of mannose and mannose-6-phosphate glycans on the enzymes (to mediate uptake via cell-surface mannose receptors), and new advances in this strategy have been few. Perhaps combining the lectin-mediated uptake capabilities of RTB with the endogenous glycans of both RTB and the payload will improve the efficiency of ERT and result in lower cost and increased efficacy.

Further experiments are necessary in order to optimize this system. Elevated expression of co-expressed carrier and payload constructs over current levels may be achieved by employing Agrobacterium-viral vectors, such as the system developed by ICON Genetics. Optimization of A. tumefaciens strain and other factors such as OD of IM cultures and incubation conditions is ongoing and is dependent on the genes being expressed. In order to facilitate the cloning of payload genes and therefore increase turn-around time, incorporation of Gateway (Invitrogen) destination sites in a Fd fusion vector is being investigated. A way to ease handling
of the various *A. tumefaciens* strains is also being investigated. This strategy relies on a stably-transformed RTB:κLC plant line, with the gene driven in plants by the T7 promoter used in bacterial systems. Under normal circumstances, RTB:κLC would not be expressed. An *A. tumefaciens* strain harboring both a Fd:payload fusion gene driven by a promoter such as CaMV 35S (or T7 promoter) and a gene encoding T7 RNA polymerase, also driven by CaMV 35S, would then be infiltrated into the RTB:κLC plant line. Expression of T7 RNA polymerase would drive expression of RTB:κLC and perhaps Fd:payload. This strategy is still in the design stage.

In summary, I have developed a plant-based flexible and efficient capture and carry RTB-mediated delivery system. Utilization of the *Agrobacterium tumefaciens*-mediated transient expression of *Nicotiana benthamiana* allows for the assembly of the scaffold that bridges the co-expressed RTB-carrier and payload proteins, which in turn facilitates easy purification through the properties of RTB. Additional experiments and controls are presently underway in order to verify and optimize the technology. Recombinant GFP is not taken up by HT-29 cells (see Figure 15), but it is necessary to show that the Fd moiety does not mediate uptake. Work performed by Jianyun Liu has shown that the anti-RTB antibody used for western immunoblots and described in Chapter 2 has a neutralizing effect on RTB-mediated delivery. We are currently performing experiments involving the use of this antibody to block uptake of RTB:κLC + Fd:GFP. Additional experiments to test this would be the purification of independently-expressed Fd:GFP or the use of crude extract samples in the uptake assay.
References


Chapter 4:
Identification and characterization of a possible proteolytic site at the N-terminus of RTB

Abstract
In the course of experiments that dealt with the engineering of RTB to be an effective protein delivery platform, a breakdown pattern among RTB-fusions was observed. Fusion constructs in which RTB was the C-terminal partner produced a large amount of a breakdown protein when transiently expressed via Agrobacterium-infiltration of N. benthamiana. This breakdown protein reacted strongly with anti-RTB antibodies, bound and eluted from immobilized lactose resin, and was of the same molecular weight as castor bean-derived and recombinant RTB. Edman degradation chemistry of breakdown proteins purified from several different infiltrated fusion constructs revealed a common N-terminal sequence (VSMDPE). We tested the hypothesis that this breakdown was the result of normal cellular proteolytic machinery responsible for the natural processing of ricin holotoxin by creating fusions to a form of RTB which was truncated by six amino acids at the N-terminus. Removal of the RTB sequence A D V C/S M D from fusion constructs resulted in elimination of cleavage at the fusion junction. In order to determine the individual amino acids which mediate this cleavage, point mutation studies were performed. When Ala 1, Asp 2 or Val 3 was changed to Gly, the cleavage event occurred, indicating that neither of these amino acids alone is responsible for the breakdown.

(Jorge Ayala of Arkanas Biosciences Institute contributed to this work by designing and performing the site-directed mutagenesis and analysis of point mutation constructs, Fig. 7)
Introduction

In the course of experiments aiming to engineer the ricin B lectin (RTB) into a flexible and efficient platform for protein delivery, a pattern of breakdown of RTB-fusion proteins recombinantly produced in *Nicotiana benthamiana* emerged. This breakdown was also observed with RTB-fusions produced in stably transformed *Nicotiana tobacum* suggesting it is not specific to either the transient expression system or to *N. benthamiana*. Fusions in which RTB constituted the C-terminal partner exhibited a near-predictable pattern, in which the large majority of lactose-binding product migrated to the same position on SDS-PAGE analysis as RTB from castor bean (cbRTB) and rRTB (~32 kD). Only a fraction of recovered recombinant protein was of the predicted molecular weight, that being the sum of the N-terminal fusion partner plus RTB. This pattern of breakdown was observed regardless of the nature of the N-terminal fusion partner; proteins as diverse as immunoglobulin (Ig) domains, interleukins, and storage protein domain (SPD), when fused to the N-terminus of RTB displayed this pattern (Reidy, Liu, Ayala, Dolan, Cramer, unpublished). Fusions in which RTB constituted the N-terminus, such as RTB:GFP (see Chapter 2) and RTB:κLC (see Chapter 3), did not display this phenomenon as obviously as when RTB was on the C-terminus. Clearly there was something about RTB, and not the fusion partner, that was mediating this breakdown.

Clues as to the possible origin of this phenomenon may be found in the post-translational processing of ricin toxin in Castor bean seeds. Figure 1 illustrates this processing. The primary gene product of the ricin toxin gene is a polypeptide chain termed preproricin. Preproricin consists of the N-terminal endoplasmic reticulum (ER)-targeting signal peptide, the A-chain N-glycosidase toxin (RTA), a short 12 amino acid linker sequence which contains vacuolar sorting information, and RTB. Following insertion into the ER and subsequent cleavage of the signal
peptide, preproricin becomes proricin: RTA fused to RTB through the 12 amino acid vacuolar-targeting linker. It is not known precisely when this linker sequence is removed completely, but it is thought to occur once in the vacuole. Following removal of the linker, separated RTA and RTB polypeptide chains are held together via a single disulfide bond and numerous hydrophobic and polar interactions. The molecular weights of both RTA and RTB are ~ 32 kD.

![Diagram of ricin processing]

Complete removal of the linker requires two distinct proteolytic events, and the nature of these cleavage events and the protease(s) responsible for them are not known. Studies by Sehnke, et al. have shown that tobacco can properly process native ricin toxin when transformed with the preproricin gene, suggesting that the proteolytic machinery necessary to remove the linker is present in plant species other than castor bean. Is it possible that this proteolytic machinery is
responsible for the described breakdown pattern of RTB-containing fusion proteins? Do fusions at the N-terminus of RTB mimic the 12 amino acid linker sequence, and therefore trigger this proteolysis? If so, this would suggest that RTB sequences serve as recognition for the protease. Thus, it is of interest to determine whether this cleavage event is precise, what constitutes the recognition site, and whether modifications of this region will result in more stable fusion products.

Here we report the characterization of the breakdown phenomenon described above, through the use of N-terminal sequencing via Edman degradation chemistry. We outline strategies for the possible reduction of the breakdown and results attained thus far.

**Methods**

**Gene construction**

Maps of the constructs used in this research are shown in Figure 2. All constructs were first created in the pBC (Stratagene) cloning vector or in a modified pBC in which the XhoI site of the polylinker had been removed (pBC-X; see Chapter 3 for details). Promoter:gene cassettes were subcloned into the pBIB-Kan binary vector\(^6\) for *Agrobacterium tumefaciens*-mediated transient expression in *N. benthamiana* via HindIII/SalI or HindIII/SacI. The map of the rRTB construct, used as a control in some studies here, can be found in Chapter 3, Figure 3 (“Construct C”). The IL10:RTB construct was a gift of Dr. Maureen Dolan (Arkansas Biosciences Institute). The SPD:RTB construct was created by Jorge Ayala (Arkansas Biosciences Institute; Ayala, Dolan, Cramer, unpublished).
Figure 2. Maps of constructs used in this research. Truncated RTB, “RTB(tr)” is denoted by the blue/black boxes. C\textsubscript{l} encodes the constant region of the mouse kappa light chain. IL-10 is the coding sequence of human interleukin-10. SPD refers to a proprietary seed storage protein domain. Z\textsubscript{spa-1} is the 58 amino acid Protein A-binding affibody developed by Gräslund, et al. Yellow boxes indicate the presence of the (Gly\textsubscript{3}Ser\textsubscript{3}) flexible linker. The patatin signal peptide is denoted by “sp”. MSP:RTB uses an signal peptide endogenous to MSP. All constructs were driven by the dual enhanced 35S CaMV promoter (de35S:TEV) for constitutive expression in plants by the Agro-mediated transient system.
Plant growth conditions, transient expression, and recovery of RTB fusions

*N. benthamiana* plants were grown and infiltrated with *Agrobacterium tumefaciens* as described in Chapter 2. Both initial enrichment and two step purification procedure for RTB are as described in Chapter 2.

Point mutations

Constructs creating point mutations within the RTB N-terminus were performed by Jorge Ayala (Arkansas Biosciences Institute), using the QuikChange II Site-directed mutagenesis kit (Stratagene, Cedar Creek TX), as per the manufacture’s instructions. The template used in the reactions was the C:L:RTB construct in pBC. The primers used in the mutagensis reaction were:

1) for RTBgdv 5’-GAGTGTCTCGAGGGTGATGTTTCTA (Forward) and 5’-CCATAGAAACATCACCTCGAACACTC (Reverse); 2) for RTBagv 5’-GAGTGTCTCGAGGCTGGTGTTTCTATGG (F) and 5’-CCATAGAAACACCAGCCTCGAGACACTC (R); 3) for RTBadg 5’-CTCGAGGCTGATGGTTTCTATGGATCC (F) and 5’-GGATCCATAGAACACCACAGCCTCGAG (R).

N-terminal sequencing

For preparation of samples, RTB-specific purified (Chapter 3) protein samples containing breakdown products to be sequenced were separated on 12% SDS-PAGE under reducing conditions and transferred to Sequi-Blot PVDF Membrane (Bio-Rad, Hercules CA). Following transfer, the membrane was stained with Coomassie and air dried. Bands to be sequenced were excised from the membrane and sent to the University of Virginia Health System Biomolecular
Results

Characterization of breakdown phenomenon

Identification, removal, and/or exploitation of a proteolytic recognition site in RTB that results in a specific pattern of cleavage of N-terminal fusion partners strengthens the utility and flexibility of a RTB-based platform for delivery of proteins. Studying this phenomenon benefited from the fact that the major breakdown product retained full lectin activity. In fact, our hypotheses that this breakdown results from the machinery responsible for proper processing of ricin toxin rests on the notion that the breakdown product represents essentially the full length RTB protein (32 kD). Enrichment and purification schemes developed in other arenas of this dissertation research provided a readily available substrate for N-terminal sequencing. N-terminal sequencing of the 32 kD breakdown protein (32BP) allowed for direct inspection of the site at which any proteolysis may have occurred. Comparing 32BPs recovered from different constructs allowed for simple characterization of the phenomenon. Investigation of a breakdown pattern in which RTB was on the N-terminus of the fusion would necessitate a different purification scheme optimization for each construct.

Before any strategies to eliminate this degradation could be designed, it was first necessary to determine the N-terminal sequence of various 32BP species derived from different constructs. We chose C\textsubscript{L}:(Gly\textsubscript{3}Ser)\textsubscript{3}:RTB (C\textsubscript{L}:link:RTB, see Chapter 3), IL10:RTB, SPD:RTB, and Z\textsubscript{spa-1}:RTB to investigate first because the fusion partners are quite different in terms of
primary structure. The IL10:RTB construct is a fusion between human interleukin 10 and RTB, a gift of Dr. Maureen Dolan (Arkansas Biosciences Institute). SPD:RTB is a fusion of RTB and a proprietary seed storage protein domain. Z_{spa-1}:RTB is a fusion between RTB and the 58 amino acid affibody that binds to Protein A, and is used in some systems as a one-step purification tag. As a control, rRTB (see Chapter 2) was included. A representation of the fusion proteins selected can be in Figure 3a, with the sequences of the patatin signal and junctions of the various fusions shown as well. The constructs were expressed in the Agrobacterium-mediated transient expression system and purified using the RTB-specific purification protocol described in Chapters 2 and 3. Empty-vector (pBK) Agrobacterium-infiltrated leaves were used as purification control. Purified proteins were separated via reducing 12% SDS-PAGE, transferred to PVDF membrane and stained with Coomassie (see Figure 3b). Analysis of RTB-purified proteins derived from plants expressing C_{L}:link:RTB (see Figure 3, lane 4) revealed three bands that reacted with anti-RTB antibodies (see Chapter 3, Figure 7, lane 4), the ~45 kD band representing full length fusion protein and two bands in the ~30 – 35 kD range. The smaller of these two bands was much higher in abundance relative to the heavier band. For IL10:RTB, two lactose-binding, anti-RTB reactive bands (data not shown) were observed for IL10:RTB, the larger intact fusion product (~ 50 kD) and the 32BP. Purified proteins from both SPD:RTB and Z_{spa-1}:RTB infiltrated plants revealed only 32BP, and no visible full-length product. N-terminal sequencing revealed bands A (rRTB), B (32BP from IL10:RTB), and C (the smaller of the two breakdown bands from C_{L}:link:RTB), E (SPD:RTB), and F (Z_{spa-1}:RTB) all contained the sequence VSMDPE. Interestingly, rRTB, that is RTB that is not fused to another protein, does not match the expected sequence. The expected N-terminus of rRTB, considering the theoretical cleavage site of the patatin signal peptide, is TSRADVSMDPE… (see Figure 3a), where the
threonine derives from the signal peptide, and the serine and arginine are derived the XbaI restriction site used to clone the gene. The sequence of band D, EGGGSG, was unexpected. This protein was termed link:RTB, as it consists of the (Gly3Ser)3 linker on the N-terminus of RTB. The glutamine residue on the N-terminus derives from the PstI site used in the cloning of this fusion. Thus, it appears that there are two breakage events occurring in Clink:RTB, one at the N-terminus of the (Gly3Ser)3 linker (band D) and another at the N-terminus of RTB (band C).

![diagram](image_url)

**Figure 3. Characterization of breakdown pattern.** A: Representation of the five proteins which produced breakdown products that were sequenced. The patatin signal peptide sequence is shown in “rRTB”. The red dot indicates the predicted signal peptide cleavage site. The sequences of the junctions between fusion partners are shown, along with the the first eight amino acids of RTB. B: Coomassie stained membranes of sequenced RTB-purified proteins. Bands A through F were N-terminally sequenced. Lane 1: Empty vector (pBK)-infiltrated leaves run through purification scheme. Lane 2: Purified rRTB. Lane 3: Purified IL10:RTB and breakdown. Lane 4: Purified Clink:RTB and breakdown bands. Lane 5: SPD:RTB. Lane 6: Zspa:RTB. C: Results of N-terminal sequencing on indicated bands. The expected RTB N-terminal sequence is at the top. Sequences of bands A, B, C, E, and F are aligned with the expected sequence. Band D sequence derives from the (Gly3Ser3) linker.

**Generation of “truncated” RTB**

The three-dimensional structure of ricin and RTB reveal that the first six amino acids of RTB form a flexible “arm” that provides proximity of RTB Cys 4 and RTA Cys 259 to form disulfide linkage. This motif is found in other type II RIPs such as mistletoe lectin I and ebulin,
even though amino acid identity is not well conserved in this region (see Figure 4a). In most of our studies, Cys 4 of RTB has been mutated to Ser (see Figure 3a), because of observed dimerization in other experiments (data not shown). This change does not impact lectin activity, and since this change is not present in the IL10:RTB construct, it did not appear to be a factor in the observed breakdown phenomenon. To ask the question of whether this flexible portion mediates breakdown, either by serving as recognition for protease(s), or by introducing energetically unstable situations, we developed and tested constructs that fused C\textsubscript{L} and C\textsubscript{L}:linker directly to Pro 7 of RTB, thus eliminating the putative AD\textsubscript{↓}VC… cleavage site. This so-called “truncated” RTB was termed RTB(tr). Pro 7 was chosen for two reasons. First, Pro 7 is the first amino acid that resides on the edge of the “core” of RTB based on the crystal structure of ricin (PDB ID# 2aai).\textsuperscript{3} Second, many proteases, such as trypsin, will not cleave peptide bonds involving proline. Figure 4b graphically represents the position of fusion proteins on the N-terminus of RTB and RTB(tr).
Impact of amino acids 1 through 6 of RTB on breakdown phenomenon

The first construct created incorporating RTB(tr) was $C_L:\text{linker}:\text{RTB(tr)}$, where “linker” is $(\text{Gly}_3\text{Ser})_3$ (see Figure 2). $C_L:\text{linker}:\text{RTB}$ and $C_L:\text{linker}:\text{RTB(tr)}$ was expressed using the Agrobacterium-mediated transient system, and leaves were collected at 24 and 48 hr. Western analysis using anti-RTB antibodies of lactose-enriched proteins revealed that at the 48 hr time
point, only one breakdown band was visible in $C_L$:linker:RTB(tr), where two were observed in $C_L$:linker:RTB, as previously demonstrated (see Figure 5a). Comparisons of lanes 4 and 6 in Figure 5a show that the breakdown band observed in $C_L$:linker:RTB(tr) migrates to a position on SDS-PAGE that is between the positions of the two breakdown bands seen in $C_L$:linker:RTB. A slightly smaller full-length $C_L$:linker:RTB(tr) compared to $C_L$:linker:RTB is also observed, consistent with the construct-mediated elimination of six amino acids. Figure 5b shows a Coomassie-stained membrane of purified $C_L$:linker:RTB(tr) (from the 48 hr time point), and it also shows only one breakdown band (compare to Figure 3b lane 4). Sequencing of Band G in Figure 5b (the breakdown product of $C_L$:linker:RTB(tr)) reveal the N-terminal sequence to be EGGGSG, the same sequence seen in Band D (the higher molecular weight breakdown band from $C_L$:linker:RTB, see Figure 3b). Band G is termed linker:RTB(tr). This is consistent with our previous result that the $(\text{Gly}_3\text{Ser})_3$ linker is susceptible to cleavage in $N.\ benthamiana$.

However, lack of other N-terminal sequences within Band G suggests that the removal of the A D V S/C M D sequence stabilizes the fusion. In order to confirm this without the “complication” of the linker-specific breakdown susceptibility, we created additional constructs lacking this...
linker (see Figure 2).

**Generation of constructs without (Gly$_3$Ser)$_3$ linker**

In order to confirm the role of this six amino acid sequence, A D V C/S M D, in mediating degradation of N-terminal fusions to RTB, constructs which omit the (Gly$_3$Ser)$_3$ linker sequence were created, termed $C_L$:RTB and $C_L$:RTB(tr) (see Figure 2). Anti-RTB western analysis comparing the lactose-binding fractions generated from the infiltration of five different constructs: an empty-vector (pBK) control, rRTB, $C_L$:link:RTB, $C_L$:link:RTB(tr), and $C_L$:RTB(tr) is shown in Figure 6. The expression of $C_L$:RTB(tr) is very low compared to the others, and a ~ 45 kD band is only visible after a long (45 min) exposure time. The fact that there is only one band present (lane 6) suggests that fusing $C_L$ to Pro 7 prevents degradation.

**Point mutations within the first three amino acids of RTB**

Results described above suggest that the first six amino acids of RTB constitute a potential proteolytic sensitive site. As a first step in further delineating this site, a series of constructs were generated that created single amino acid replacements within this region. Point mutations in which the first, second, or third amino acid of RTB in the $C_L$:RTB construct was changed to glycine were created to determine if any of these individual amino acids are critical to
the observed degradation. The specific changes are delineated in Figure 7a. Anti-RTB Western analysis of lactose-binding fractions generated from these constructs can be seen in Figure 7b. All three constructs produced identical patterns of degradation, indicating that the first three amino acids of RTB are not individually responsible for the observed breakdown. N-terminal sequencing of these 32bp species has not yet been completed.

Discussion

We have reported the preliminary characterization of a phenomenon in which proteins fused to the N-terminus of RTB are cleaved off in a relatively predictable manner. It has been shown that the first six amino acids of RTB, A D V C/S M D, facilitate this breakdown. These studies have been somewhat complicated by the observation that the (Gly$_3$Ser)$_3$ linker sequence used to provide spacing between fusion partners in other work is also susceptible to distinct cleavage events. RTB fusion products in which the first six amino acids surrounding the RTB cleavage event (i.e., from constructs C$_L$:link:RTB(tr) and C$_L$:RTB(tr)) were not cleaved within the RTB N-terminal domain, although cleavage upstream of the (Gly$_3$Ser)$_3$ linker was still observed. The low expression of C$_L$:RTB(tr) is disappointing, but efforts are underway to
address this problem and to determine if the low expression level observed results directly from
the absence of A D V C/S M D. This is unlikely since absence of A D V C/S M D in
C_L:link:RTB(tr) does not seem to affect expression level.

With the exception of link:RTB and link:RTB(tr), all 32BP species sequenced contained
VSMDPE as a major or minor sequence. Other sequences identified were ADVSMD
(IL10:RTB), SMDPE (C_L:link:RTB), and DVSMSP (SPD:RTB). These slight variations may
be the result of the influence of the N-terminal partner on the protease.

The specific cleavage within the N-terminus of the RTB fusion partner could result from
protease activity or from energetically unfavorable conformations in this region. The
observation that rRTB, a construct in which no fusions are made to RTB, also displays the
degradation, (that is, it has the same N-terminal sequence as products derived from the
breakdown of fusion proteins) suggests that this cleavage is not the result of physical constraints
due to energetically unfavorable fusion partner pairings. Similarly, the 32BP comprised the
major product of constructs where RTB was fused to a highly flexible (Gly_3Ser)_3 linker
suggesting that steric or conformational stresses are unlikely to account for this cleavage. The
question of whether the breakdown is indeed due to protease activity could be addressed through
the use of various protease inhibitors. Broad range inhibitors such as PMSF, leupeptin, etc.
included in the infiltration media may be used to help determine the class of the protease(s)
involved. The fact that not all lectin-positive, anti-RTB reactive bands are degraded (for
example, a significant amount of full-length C_L:link:RTB is recovered) suggests that perhaps this
degradation is occurring in one specific location along the secretory pathway. Full-length
species may be the result of proteins that have not yet reached the point in the pathway where the
mystery protease(s) resides, or have passed through the compartment without interacting with the
protease. To test this, it may be possible to stop movement within the secretory pathway by use of chemicals such as brefeldin A, which interferes with protein movement from the ER to the Golgi. Infiltration of brefeldin A during or at some point after the *Agrobacterium* is introduced may provide insight into where the cleavage is occurring.

We are interested in delineating key components of the “recognition domain” for this cleavage event. As a first step we utilized site-specific mutagenesis to alter the first three amino acids surrounding the common cleavage site (A D ↓ V C/S M D ) in RTB. Although we do not yet know the precise N-terminal residues of these products, as shown in Figure 7, none of these alterations prevented cleavage, suggesting that the “recognition domain” may be structural or reside further downstream in the RTB N-terminal domain. In order to address this, we have begun designing constructs that will place the A D V C/S M D sequence between two unrelated proteins. It is important to use as the C-terminal partner a protein that is easily enriched or purified through a well-defined chromatography regime, for subsequent N-terminal sequencing analysis. It is possible that the breakdown phenomenon observed relies more on secondary and tertiary structure than primary sequence. This remains to be elucidated.

Pursuing this cleavage is advantageous for several reasons. First, eliminating a known proteolytic site increases the utility of the RTB-based delivery system in situations where stable fusions at the N-terminus of RTB are required. This is particularly important in situations where RTB needs to be the C-terminal partner, for example, where the payload requires an unobstructed N-terminus for activity. Second, by identifying both the recognition sequence and the protease, it may be possible to incorporate this cleavage into other recombinant production systems where removal of a purification tag is desired. For example, cleavage sites for thrombin, enterokinase and tobacco etch virus (TEV) proteases are widely used in strategies for producing
recombinant fusion proteins. This work is still ongoing, and conclusions made here are subject to change dependent upon new data collected in the future.
References


Figure 7, page 96, includes a Western blot performed by Jorge Ayala, MS, Research Associate at Arkansas Biosciences Institute (2006). Used by permission.