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Finding a new vaccine in the ricin protein fold

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Previous attempts to produce a vaccine for ricin toxin have been hampered by safety concerns arising from residual toxicity and the undesirable aggregation or precipitation caused by exposure of hydrophobic surfaces on the ricin A-chain (RTA) in the absence of its natural B-chain partner. We undertook a structure-based solution to this problem by reversing evolutionary selection on the 'ribosome inactivating protein' fold of RTA to arrive at a non-functional, compacted single-domain scaffold (sequence RTA1–198) for presentation of a specific protective epitope (RTA loop 95–110). An optimized protein based upon our modeling design (RTA1–33/44–198) showed greater resistance to thermal denaturation, less precipitation under physiological conditions and a reduction in toxic activity of at least three orders of magnitude compared with RTA. Most importantly, RTA1–198 or RTA1–33/44–198 protected 100% of vaccinated animals against supra-lethal challenge with aerosolized ricin. We conclude that comparative protein analysis and engineering yielded a superior vaccine by exploiting a component of the toxin that is inherently more stable than is the parent RTA molecule.

Keywords: protein aggregation/protein engineering/ribosome inactivating proteins

Introduction

Globular proteins can assume useful metastable conformations on folding pathways or for specific biological functions, including membrane association or translocation (Carr *et al.*, 1997; Shin *et al.*, 1997; Gorovits *et al.*, 1998; Sohl *et al.*, 1998; Jaswal *et al.*, 2002). Outside natural environments, however, these states often present a barrier to the efficient production of biopharmaceuticals because exposure of hydrophobic surfaces contributes to instability or aggregation that reduces product yield and, under conditions of over-expression in prokaryotic systems, can lead to sequestration in inclusion bodies (Wilkinson and Harrison, 1991; De Bernardez Clark *et al.*, 1998; Hammarstrom *et al.*, 1999). Developing means to control aggregation is of fundamental importance in modulating the function of certain proteins in conformational disease states, and also for the efficient production of protein vaccines or therapeutics (Carrell and Lomas, 1997; Wanker, 2000; Carrotta *et al.*, 2001; Millard *et al.*, 2003). Designing protein stability or solubility remains a significant protein engineering challenge because aggregation often involves relatively large, discontinuous and transiently exposed interfacial regions among different subunits or other binding partners.

Ricin, a widely available toxin from the common castor bean plant (*Ricinus communis*), is composed of two different protein subunits linked by a single disulfide bond. The ricin B-chain (RTB) is a lectin that binds galactose or *N*-acetylgalactosamine receptors on the surface of target cells to promote endocytosis and trafficking of the toxin to the trans-Golgi. The ricin A-chain (RTA) is a highly efficient *N*-glycosidase ($k_{cat}/K_m \approx 10^7$ M^{-1} s^{-1}) (Endo and Tsurugi, 1988) that enters the cytosol of target cells by adopting a transient, metastable state to exit the Golgi (Argent *et al.*, 2000). Once inside the cell, RTA acts as a 'ribosome inactivating protein' (RIP) to remove a specific adenine base of the essential 28S ribosomal RNA, effectively stopping new protein synthesis, inducing apoptosis and killing eukaryotic cells (reviewed in Lord *et al.*, 2003).

The potency of RTA has found beneficial application in the field of chemotherapy as a component of 'immunotoxin' conjugates, but the toxin also has been exploited as a poison for biological warfare and bioterrorism (Franz *et al.*, 1997). Inhalation of small amounts of ricin aerosol can rapidly and irreversibly damage cells of the respiratory tract, leading to severe pulmonary incapacitation or death (Olsnes *et al.*, 1982; Griffiths *et al.*, 1996). The lack of specific medical treatment options for ricin exposure has led to a worldwide search for effective vaccine immunogens (Hewetson *et al.*, 1993; Griffiths *et al.*, 1996, 1999; Smallshaw *et al.*, 2003). Improved understanding of the immune response to RTA also may lead to new means of eliminating the systemic toxicity that has been observed during the clinical application of RTA immunotoxins (O'Toole *et al.*, 1998; Schindler *et al.*, 2001).

Past attempts to develop a ricin vaccine suggest that, although isolated RTA can induce protective immunity against the toxin in animals, the use of RTA as a vaccine component is limited by its potentially toxic RIP activity and the undesirable tendency of RTA to self-aggregate in the absence of RTB (Piatak *et al.*, 1988). Active-site substitutions have been proposed as a means of inactivating RTA and rendering it safe for use in a ricin vaccine (Ready *et al.*, 1991; Roberts *et al.*, 1992; Smallshaw *et al.*, 2002). Although effective at reducing RIP activity, this approach does not address the problem of precipitation during production or storage of RTA as a biopharmaceutical. To overcome both problems simultaneously, wide-scale modifications to the RIP protein fold itself were undertaken. We report here on the conversion of RTA to a single-domain structure that is more stable than the parent molecule and demonstrate that the new scaffold suffices to produce safe and effective vaccine candidates.

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14. ABSTRACT

Previous attempts to produce a vaccine for ricin toxin have been hampered by safety concerns arising from residual toxicity, and the undesirable aggregation or precipitation caused by exposure of hydrophobic surfaces on the ricin A-chain (RTA) in the absence of its natural B-chain partner. We undertook a structure-based solution to this problem by reversing evolutionary selection on the "ribosome inactivating protein" fold of RTA to arrive at a non-functional, compacted single-domain scaffold (sequence RTA1-198) for presentation of a specific protective epitope (RTA loop 95-110). An optimized protein based upon our modeling design (RTA1-33/44-198) showed greater resistance to thermal denaturation, less precipitation under physiological conditions, and a reduction in toxic activity of at least three orders of magnitude compared with RTA. Most importantly, RTA1-198 or RTA1-33/44-198 protected 100% of vaccinated animals against supra-lethal challenge with aerosolized ricin. We conclude that comparative protein analysis and engineering yielded a superior vaccine by exploiting a component of the toxin that is inherently more stable than is the parent RTA molecule.

15. SUBJECT TERMS

ricin, vaccine, single-domain, A chain, recombinant, stability

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Materials and methods

Computational analysis of structure

A structure–structure alignment between RTA and pokeweed antiviral protein (PAP) was performed to determine the relative hydrophobicity of the C-terminal regions. This allowed us to assess possible differences in solubility of the individual protein chains. The electrical potentials for RTA and PAP projected on to their molecular surfaces were calculated to highlight differences in solvent polarization of the C-terminal regions. Electrostatic solvation free energies were calculated from the Poisson–Boltzmann equation using a charging–uncharging process (Nicholls *et al.*, 1991; Olson and Cuff, 1999) and the secondary structure and topology of RTA were analyzed for variations in protein compactness. Reaction field energies were determined for each protein by using a protein dielectric constant of 2, while the implicit solvent was treated by using a dielectric constant of 80.

DNA cloning

Cloning was based on the published gene sequence of RTA (Lamb *et al.*, 1985). To prepare the RTA truncation constructs, a 'stop codon' was incorporated at the desired position by mutagenesis. Ten amino acid residues at positions RTA 34–43 were removed using the polymerase chain reaction method to produce RTA1–33/44–198. DNA sequencing of all RTA derivatives was performed to confirm that only the desired changes had been introduced in the gene sequence. The DNA products were purified, ligated to a commercial expression vector based upon the T7 promoter system (Studier *et al.*, 1990) and then used to transform competent *Escherichia coli* BL21 DE3 cells (Invitrogen, Carlsbad, CA).

Protein expression and purification

Transformed cells were grown in TB media containing kanamycin (50 μg/ml) until reaching a cell density of 0.4–0.6 OD_{600}. Induction of genes expressing RTA sequences was performed using IPTG (1 mM) at 25°C, for 18–20 h.

To evaluate protein levels and the extent of aggregation during expression, cell extracts were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and immunoblotting with a previously characterized polyclonal antisera. Briefly, cells were solubilized in 50 mM sodium phosphate buffer, pH 7.3 and the resulting buffer-insoluble pellet was boiled in 6 M urea, pH 7.

Protein purification

Cell paste was dissolved in ice-cold 50 mM sodium phosphate buffer, 2 mM EDTA, pH 7.3 at a ratio of 1 g wet weight/15 ml buffer. Cells were disrupted by sonication. Homogenized cells were centrifuged using a Sorvall SS-34 rotor at 15 000 r.p.m. for 15 min at 4°C. Proteins were purified from the supernatant by a combination of conventional ion-exchange separations using commercial Mono-Q and Mono-S 10/10 columns (Pharmacia, Piscataway, NJ) and hydrophobic interaction chromatography.

Characterization of purified product

Fractions containing the RTA derivatives at >95% purity as judged by Coomassie Brilliant Blue-stained gels were pooled. Buffer was changed to 120 mM NaCl, 2.7 mM KCl, 10 mM $NaPO_4$, pH 7.4 [phosphate-buffered saline (PBS)] by dialysis and purified protein was filtered (0.2 μm pore size) and stored sterile at 4°C prior to animal studies. The identity of purified proteins was confirmed by immunoblots with polyclonal antiserum and by Edman N-terminal degradative sequencing.

As a control for comparison with the vaccine candidates, recombinant (r) RTA was expressed and purified essentially as described (O'Hare *et al.*, 1987; Piatak *et al.*, 1988; Ready *et al.*, 1991).

Residual RIP activity

Toxic *N*-glycosidase activity was assessed by the ability of purified proteins to block luciferase synthesis in a commercial cell-free translation assay as described (Hale, 2001). Assays were calibrated using known amounts of purified, natural ricin (Vector, Burlingame, CA) or rRTA.

Dynamic light scattering

A Dyna-Pro MS800 instrument (Protein Solutions) was used to make right-angle dynamic laser-light scattering measurements on protein solutions at 0.8 mg/ml in PBS at 25°C. Molecular weights were estimated using the standard curve for small globular proteins contained in the Dynamics software package.

Circular dichroism (CD)

A Jasco-810 spectropolarimeter was used to take CD spectra in the far-UV region on protein solutions at 0.2 mg/ml in a stoppered 1 mm pathlength cuvette. Heating was carried out at 1 K min^{-1} and data were not smoothed.

Protein precipitation time course

Samples of rRTA and/or RTA1–33/44–198 at 0.2 mg/ml in PBS were incubated at 37°C and centrifuged at each time point to remove insoluble denatured protein. The total remaining soluble protein at each time point was measured by Bio-Rad protein dye assay and SDS–PAGE.

Vaccine studies

A mouse model was used to evaluate the efficacy of the recombinant, RTA-derived polypeptides. Mice were vaccinated intramuscularly at 0, 4 and 8 weeks with 10 μg of the test polypeptide ± adjuvant. Three weeks after the last vaccination, blood was obtained for serology and 1 week later the mice were challenged with 10 LD_{50} or 5 LD_{50} of ricin by intraperitoneal injection or by whole-body aerosol in a class III hood line. In separate experiments, dose–responses to the vaccine candidates were determined by immunizing with increasing amounts of protein immunogen, followed by ricin challenges by whole-body aerosol or intraperitoneal injection.

Results and discussion

RTA and other RIPs achieve catalysis upon a multi-domain, αβ protein fold that occurs naturally in a variety of structural assemblages including monomers (e.g. PAP and the MAP-30 anti-HIV protein), AB heterodimers (e.g. ricin, abrin and modeccin toxin) or AB5 multimers (e.g. *Shigella dysenteriae* toxin and Shiga-like bacterial toxins) (Villafranca and Robertus, 1981; Montfort *et al.*, 1987; Katzin *et al.*, 1991; Robertus, 1991; Rutenber *et al.*, 1991; Monzingo *et al.*, 1993; Weston *et al.*, 1994). The observed diversity in RIP architecture can be understood as a consequence of sequence fitness of protein structures constrained by thermodynamics and function.

If one 'threads' a sequence through a three-dimensional protein structure, a thermodynamic profile emerges where certain regions are stable and hence have high sequence compatibility scores, while other regions are less favorable. Sequence–structure fitness can be exploited for protein engineering by analyzing residue propensities for particular fold types, compactness, hydration and conformational entropy, among other terms.

An examination of the RTA–RTB interfacial region with the equivalent region of monomeric PAP revealed that the RTA sequence contains significantly more hydrophobic residues (~30% versus 15%). This increase in hydrophobic character leads to an electrical potential of the RTA region that is less favorable in terms of hydration than is the corresponding PAP region (see Figure 1A). In other words, the release of water molecules at the RTA interface strongly favors the reassociation with RTB or RTA self-aggregation, whereas PAP is more hydrophilic. The notion of hydration preference was corroborated by numerical solution to the Poisson–Bolztmann equation for the calculation of the free energy of electrostatic charging of RTA or PAP in a low-dielectric medium surrounded by a high-dielectric solvent environment.

Our comparative analysis pointed to a partitioning of function between the two domains of RTA, with the N-terminal sequence of RTA optimized to serve as an 'anchoring' fold upon which RIP catalytic activity may have evolved by adaptive radiation. In this model, the C-terminal RTA domain, comprising approximately residues RTA 200–267, is a later functional modification that contributes to RIP activity and in two-chain RIPs, also provides the hydrophobic interfacial region with the lectin subunit (Figure 1B). An additional function of the C-terminus may be to facilitate membrane translocation of RTA through the adoption of metastable states with exposed hydrophobic surfaces (Chaddock *et al.*, 1995, 1996; Day *et al.*, 1996; Beaumelle *et al.*, 1997; Argent *et al.*, 2000). We chose to exploit this structural hierarchy of ricin by splitting the functions to eliminate the undesirable hydrophobic surface of the C-terminus, while preserving a previously identified neutralizing epitope (Aboud-Pirak *et al.*, 1993; Lebeda and Olson, 1999).

Based on the globular organization and compactness of RTA, several regions in the structure were carefully evaluated for separating the two domains. The coiled 'linker' region

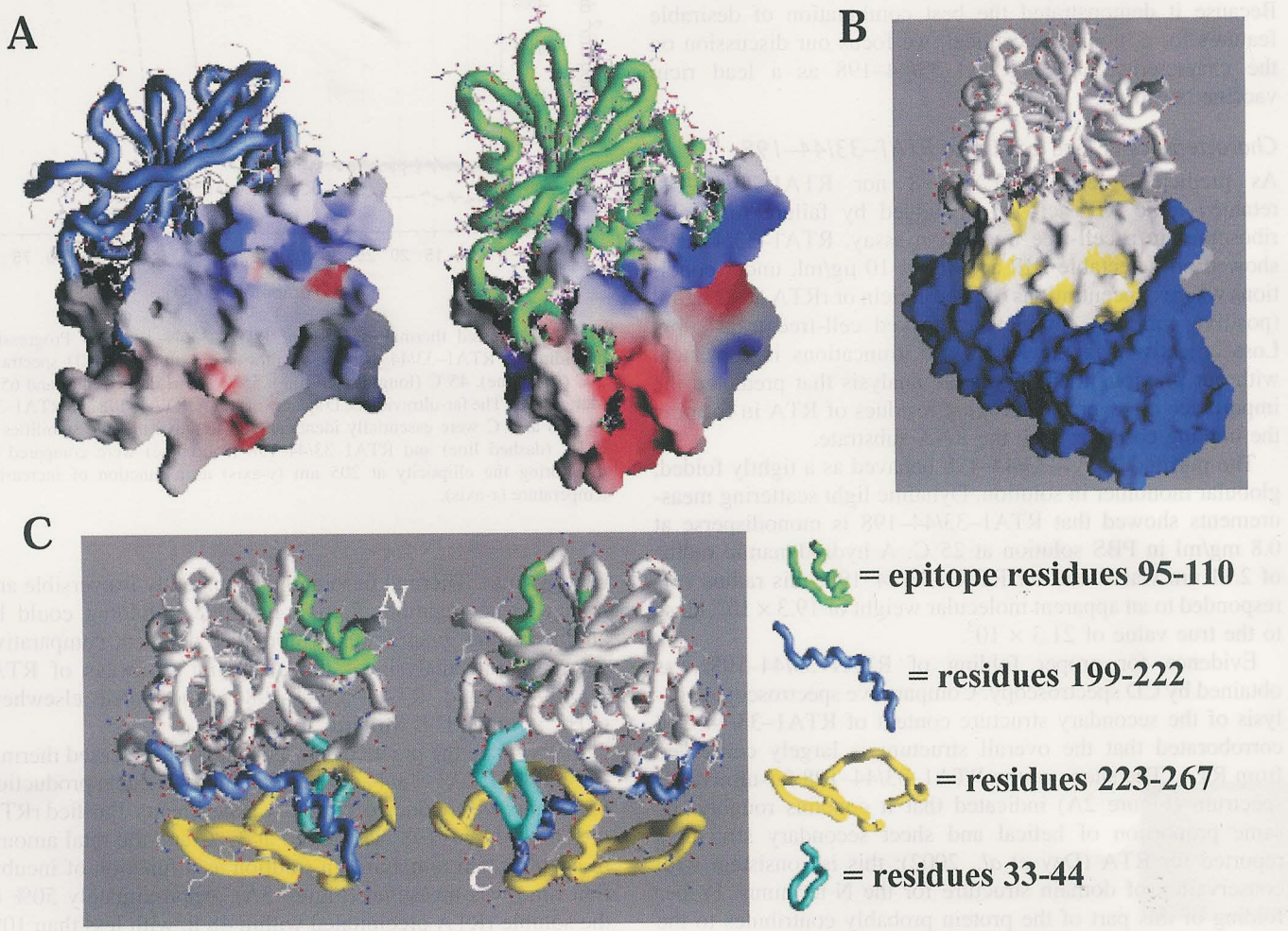

Fig. 1. (**A**) Comparison of the calculated electric field potentials for the C-terminal domains generated by RTA (left) and PAP (right) proteins projected on to their molecular surfaces. The red surface elements are negatively charged regions and blue are positively charged regions. (**B**) Ricin holotoxin structure emphasizing the close association between RTB (dark blue) and RTA (white ribbon). Molecular surfaces for RTB and part of RTA are shown to highlight the extent of hydrophobic packing between subunits. (**C**) Ribbon diagram of RTA depicting key structural regions modified during the course of engineering the vaccine. Parts of RTA colored blue and yellow interact with RTB in the holotoxin and were deemed undesirable in arriving at a stable fold for the vaccines. The important neutralizing epitope we identified previously is colored green (Aboud-Pirak *et al.*, 1993; Lebeda and Olson, 1999). The lead vaccine construct is colored white and green with loop closure at residues 33 and 44.

between residues RTA 190 and 198 was selected because: (1) it appeared hydrophilic, favoring both conformational flexibility and solvent polarization; (2) it avoided inclusion of the backbone-torsional restrictive residue, Pro200, which was expected to interfere with compact folding of the residual C-terminus; and (3) computer models of the RTA–RNA assembly predicted that removing the entire fragment 199–267 from the RTA fold would eliminate critical residues required for high-affinity binding of the RNA substrate, including Asn209, Trp211 and Arg213 (Olson, 1997; Olson and Cuff, 1999).

From modeling solvent effects for the truncated RTA1–198, we noticed that water was now predicted to fill several protein cavities not initially exposed to solvent in RTA. One structural element of concern was a loop region (RTA 34–43) which unfavorably increased overall solvent accessibility of the protein. We conjectured that removing this loop from the RTA1–198 platform to create the polypeptide RTA1–33/44–198 should further optimize compactness of the structure, thereby disfavoring protein unfolding and aggregation.

To test our hypothesis, we expressed and purified RTA1–222, RTA1–198, RTA1–33/44–198 and related RTA derivatives (Figure 1C). RTA1–222 was found to be less soluble during purification than were the RTA1–198 truncations. Because it demonstrated the best combination of desirable features for a biopharmaceutical, we focus our discussion on the characterization of RTA1–33/44–198 as a lead ricin vaccine construct.

Characteristics of lead candidate RTA1–33/44–198

As predicted, neither RTA1–198 nor RTA1–33/44–198 retained toxic RIP activity as judged by failure to inhibit ribosomes in a cell-free translation assay. RTA1–33/44–198 showed no detectable RIP activity at 10 μg/ml, under conditions where concentrations of natural ricin or rRTA at <2 ng/ml (positive controls) completely blocked cell-free translation. Loss of activity in the RTA1–198 truncations is consistent with our previous thermodynamic analysis that predicted the importance of several C-terminal residues of RTA in forming the binding complex with the RNA substrate.

The purified RTA1–33/44–198 behaved as a tightly folded, globular monomer in solution. Dynamic light scattering measurements showed that RTA1–33/44–198 is monodisperse at 0.8 mg/ml in PBS solution at 25°C. A hydrodynamic radius of 2.11 nm was found for RTA1–33/44–198; this radius corresponded to an apparent molecular weight of 19.3×10^3, close to the true value of 21.3×10^3.

Evidence for proper folding of RTA1–33/44–198 was obtained by CD spectroscopy. Comparative spectroscopic analysis of the secondary structure content of RTA1–33/44–198 corroborated that the overall structure is largely conserved from RTA. The shape of the RTA1–33/44–198 far-ultraviolet spectrum (Figure 2A) indicated that it contains roughly the same proportion of helical and sheet secondary structures reported for RTA (Day *et al.*, 2002); this is consistent with conservation of domain structure for the N-terminus. Proper folding of this part of the protein probably contributes to the correct presentation of the neutralizing epitope, RTA95–110.

The most compelling evidence that our selective sequence modifications to RTA have indeed converted it to a more stable fold is found in thermal melt experiments. As shown in Figure 2B, RTA1–33/44–198 is more resistant to thermal denaturation than is the parent RTA molecule by ~7 K (1 K/min heating rate). Thermal unfolding is apparently irreversible and linked to aggregation. Partly reversible unfolding could be attained using guanidinium chloride; a detailed, comparative spectroscopic analysis of the unfolding pathways of RTA, RTA1–198 and RTA1–33/44–198 is presented elsewhere (J.H.Carra and C.B.Millard, submitted).

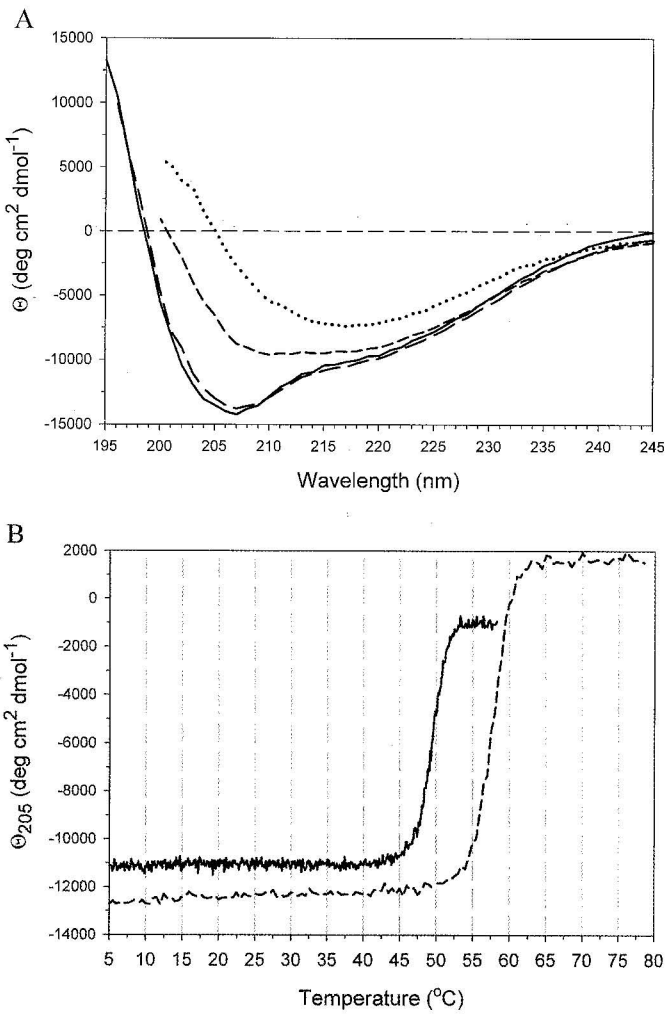

Fig. 2. Enhanced thermal stability of RTA1–33/44–198. (**A**) Progressive unfolding of RTA1–33/44–198 by monitoring far-ultraviolet CD spectra at 5°C (solid line), 45°C (long dashed line), 55°C (short dashed line) and 65°C (dotted line). The far-ultraviolet CD spectra of rRTA, RTA1–198 and RTA1–33/44–198 at 5°C were essentially identical. (**B**) Relative thermal stabilities of rRTA (dashed line) and RTA1–33/44–198 (solid line) were compared by monitoring the ellipticity at 205 nm (*y*-axis) as a function of increasing temperature (*x*-axis).

To evaluate the practical consequences of increased thermal stability of RTA1–33/44–198 during future vaccine production or storage, we conducted solubility experiments. Purified rRTA or RTA1–33/44–198 was placed at 37°C and the total amount of each protein remaining in solution as a function of incubation time was measured (Figure 3A). Approximately 50% of the soluble rRTA precipitated within 48 h, with less than 10% of starting protein left in solution at 106 h. In contrast, about 60% of the RTA1–33/44–198 remained in solution at 106 h. To visualize the extent of aggregation and to rule out contamination of either sample with proteases, we also incubated the two proteins together and analyzed soluble protein by SDS–PAGE (Figure 3B).

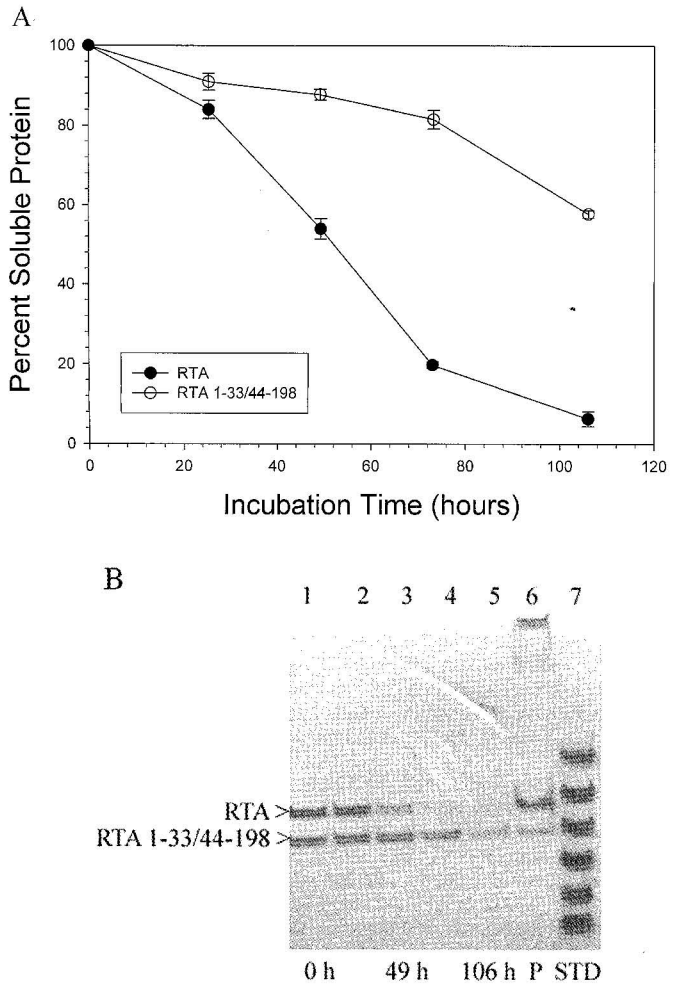

Fig. 3. Reduced tendency of RTA1–33/44–198 to precipitate compared with rRTA. (**A**) Percentage of protein remaining in solution as a function of time in PBS at 37°C. (**B**) Equal amounts of rRTA (0.1 mg/ml) and RTA1–33/44–198 (0.1 mg/ml) were mixed and incubated at 37°C. At various time points, the sample was centrifuged to pellet insoluble material. Aliquots of soluble protein were removed and analyzed by SDS–PAGE. Lane 1, 0 h; lane 2, 25.5 h; lane 3, 49 h; lane 4, 73 h; lane 5, 106 h; lane 6, insoluble pellet (P) after 106 h; lane 7, molecular weight standards.

When administered according to a qualified intramuscular vaccination protocol, purified RTA1–33/44–198 protected 10 of 10 mice from a whole-body aerosol challenge with ricin at 5–10 times the amount of toxin required to kill 50% of control, unprotected animals (LD_{50}). Vaccinated mice survived with or without co-administration of an adjuvant (Table I). In separate dose–response and potency studies, as little as 1.25 μg/mouse of RTA1–33/44–198 protected against five times the LD_{50} of inhaled ricin. At 20 μg/mouse, RTA1–33/44–198 plus adjuvant protected 100% of mice against 30 times the LD_{50} of inhaled ricin. Vaccination with purified RTA1–33/44–198 resulted in no toxicity in two animal models (not shown), consistent with the loss of potentially toxic RIP activity measured *in vitro*.

Purified RTA1–33/44–198 could be frozen and thawed without losing its efficacy as a vaccine and, unlike recombinant RTA, which visibly precipitated upon overnight storage in phosphate buffer at pH 6 during purification, there was no loss of solubility observed for RTA1–33/44–198 during protein purification. Moreover, RTA1–33/44–198 could be stored for months at >5 mg/ml in physiological saline solutions with no detectable decrease in biological potency.

Table I. Recombinant RTA1–198 or RTA1–33/44–198 immunogens protect mice from lethal exposure to ricin toxin

Treatment	Dose[a]	Survival (alive/total)	Mean time to death (days)
Intraperitoneal injection of 10 times the LD_{50} of ricin toxin			
RTA1–198	10 μg	9/10[b]	1.67
RTA1–198 + 0.2% Alhydrogel	10 μg	10/10[b]	>14
RTA1–33/44–198	10 μg	10/10[b]	>14
RTA1–33/44–198 + 0.2% Alhydrogel	10 μg	10/10[b]	>14
PBS (control)	0.1 ml	0/10	0.74 ± 0.01[c]
Aerosol whole-body exposure to between 5 and 10 times the LD_{50} of ricin toxin			
RTA1–198	10 μg	10/10[b]	>14
RTA1–198 + 0.2% Alhydrogel	10 μg	10/10[b]	>14
RTA1–33/44–198	10 μg	10/10[b]	>14
RTA1–33/44–198 + 0.2% Alhydrogel	10 μg	10/10[b]	>14
PBS (control)	0.1 ml	0/10	0.83 ± 0.02[c]

[a]Three intramuscular injections at 0, 4 and 8 weeks.
[b]Significantly different ($P < 0.01$) from PBS controls by Fisher's exact test.
[c]Average ± standard error.

Comparison with other ricin vaccine candidates

Vitetta and colleagues proposed a recombinant RTA vaccine that combines active-site substitutions to reduce RIP activity with the removal of a buried 'vascular leak peptide' (VLP) sequence (Smallshaw *et al.*, 2002). The VLP has been implicated in a complex pulmonary vascular leak syndrome reported in cancer patients administered active RTA immunotoxins (Soler-Rodriguez *et al.*, 1992, 1993; Schnell *et al.*, 2003). Although RTA1–33/44–198 retains the VLP sequence, it did not cause weight loss in vaccinated animals or other signs of vascular leak syndrome. This may be explained by the elimination of RIP activity from RTA1–33/44–198, and/or by the relatively small amounts and rapid clearance of RTA immunogens compared with RTA–antibody conjugates (Smallshaw *et al.*, 2002). Biological activity of the buried VLP also may require RTA to partly unfold or undergo other structural reorganization dependent upon the C-terminal domain that is lacking in the RTA1–198 proteins. We are currently testing this hypothesis by application of the purified RTA1–198 proteins to a human endothelial cell model *in vitro*.

The conventional protein engineering approach of eliminating activity by introducing single amino acid substitutions can be problematic for ricin because of the resilient plasticity of the RTA active site in obtaining the catalytic transition state. For example, both Glu177 and Arg180 are invariant residues among known RIPs and are thought to play crucial roles in the catalytic mechanism (Monzingo and Robertus, 1992; Chaddock and Roberts, 1993). Disrupting the ion pair between Glu177 and Arg180 at the RTA active site, however, often reduces but does not eliminate RIP activity. The Glu177 to Ala177 mutation demonstrated a remarkable rescue of electrostatic balance in the active site, achieved by the rotation of a proximal Glu208 into the space vacated by Glu177 (Kim *et al.*, 1992). Likewise, Arg180 to His180 reduces RIP activity over 500-fold, yet it remains cytotoxic (Day *et al.*, 2002).

Additionally, as noted by Robertus and co-workers, RTA Glu177 to Gln177 retains residual RIP activity and it appears to be far less well behaved than RTA in terms of expression levels (Schlossman *et al.*, 1989; Ready *et al.*, 1991).

The RTA1–198 truncations inherently lack several active-site residues, including Glu208, Asn209, Trp211 and Arg213. In separate experiments, we also attempted to inactivate RTA by single substitutions of active-site residues, including removal of the charge at positions Glu177, Arg180, Glu208 or Arg213. Consistent with previously published reports, however, we found that these proteins either were expressed at low yield or were expressed predominantly in the insoluble fraction following cell disruption (not shown). Given the poor results with expressing these individual RTA active-site mutants and the complete loss of *N*-glycosidase activity in RTA1–198 truncations, we did not attempt to include Glu177 or Arg180 mutations in the RTA1–198 platform.

Mutational and modeling studies of RTA and its binding to RNA suggest why isolated active-site substitutions often fail to produce structurally robust immunogens (see, for example, Olson and Cuff, 1999; Marsden *et al.*, 2004). We propose that self-organization of the native RTA tertiary fold is optimized by the electrostatic charge balance of the active-site cavity (Olson, 2001). In other words, altering the charge balance consequently leads to structural reorganization, coupled with a reduction in stability of the protein fold. Therefore, rather than simply introducing site-specific change(s) to eliminate activity, we altered the RIP fold by dedifferentiation of the molecule to achieve a more stable structure. Because it completely lacks catalytic activity and still protects as a vaccine, the designed fold may be of wider utility in devising vaccines for other deadly RIP toxins such as abrin or modeccin.

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