Identification of a novel functional domain of ricin responsible for its potent toxicity
Jianxing Dai1,2,5, Lei Zhao1,5, Haiou Yang1,3,5, Huaizu Guo3, Kexing Fan1,2, Huaqing Wang1,2,
Weizhu Qian1,2, Dapeng Zhang1,2, Bohua Li1,2, Hao Wang1,2 and Yajun Guo1,2,3,4
1 International Joint Cancer Institute, The Second Military Medical University,
Shanghai 200433, People’s Republic of China;
2 National Engineering Research Center for Antibody Medicine, State Key Laboratory of
Antibody Medicine and Targeting Therapy and Shanghai Key Laboratory of Cell
Engineering, Shanghai 201203, People’s Republic of China
3 School of Medicine and School of Pharmacy, The Center for Antibody Medicine of
Ministry of Education, Shanghai Jiao Tong University, 227 South Chongqing Road,
Shanghai 200025, People’s Republic of China
4 PLA General Hospital Cancer Center, PLA General Hospital, Beijing 100853, People’s
Republic of China
5 J.D., L.Z, and H.Y. contributed equally to this work.

Running title: The flexibility of an α-helix regulates the catalytic activity of ricin
Address correspondence to: Yajun Guo: International Joint Cancer Institute, The Second
Military Medical University, 800 Xiang Yin Road, Shanghai 200433, People’s Republic of
China. Phone: 86-21-81870801; Fax: 86-21-65306667; e-mail: yjguo@smmu.edu.cn

Ribosome-inactivating proteins (RIPs) are toxic N-glycosidases that depurate the
universally conserved α-sarcin loop of large rRNAs. It has received attention in biological
and biomedical research due to their unique biological activities towards animals and
human cells as cell-killing agents. A better understanding of the depurination
mechanism of RIPs could allow us to develop potent neutralizing antibodies and to design
efficient immunotoxins for clinical use. Among these RIPs, ricin exhibited
remarkable efficacy in depurination activity and highly conserved tertiary structure with
other RIPs. It can be considered as a prototype to investigate the depurination
mechanism of RIPs. In the present study, we successfully identified a novel functional
domain responsible for controlling the depurination activity of ricin, which is located
far from the enzymatic active site reported previously. Our study indicated that ricin
A-chain (RTA) mAbs binding to this domain (an α-helix comprising the residues 99-106)
exhibited unusual potent neutralizing ability against ricin in vivo. To further investigate the
potential role of the α-helix in regulating the catalytic activity of ricin, RTA variants with
different flexibility of the α-helix were rationally designed. Our data clearly
demonstrated that the flexibility of the α-helix is responsible for controlling the depurination
activity of ricin and determining the extent of protein synthesis inhibition, suggesting that
the conserved α-helix might be considered as a potential target for the prevention and
treatment of RIP poisoning.

Ribosome-inactivating proteins (RIPs) are depurinating rRNA N-glycosidases (E.C.
3.2.2.22) that cleave a single bond between a specific adenine and ribose of rRNA in
eukaryotes (1). They are generally divided into two classes (1-3). Type I RIPs such as
trichosanthin and gelonin are monomeric enzymes of approximately 30 kDa. Type II RIPs
are heterodimeric proteins with an approximate molecular weight of 60 kDa, in which one
polypeptide with RIP activity (A-chain) is linked by a disulphide bridge to a galactose-binding
lectin (B-chain). The B-chain is able to bind to a

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galactose-containing receptor on the surface of sensitive cells and mediate transport of the A-chain through the secretory pathways into the cytoplasm.

Ricin (a type II RIP), which has emerged as a powerful catalyst for mammalian ribosomes, is a good prototype to investigate the N-glycosidase mechanism of RIPs. Ricin A-chain (RTA) is the catalytic subunit of ricin, which catalyzes the depurination of an invariant adenosine residue, A\textsubscript{4324}, within the GA\textsubscript{4324}GA tetraloop motif of the highly conserved sarcin-ricin loop of eukaryotic 28S rRNA (4). Most of previous studies have focused on the role of the active-site residues that are crucial for catalytic activity of RIPs. Day and his colleagues have presented the crystal structure of ricin, indicating that RTA has a prominent cleft able to recognize the target rRNA stem-loop (5). Site-directed mutagenesis, as well as analysis by systematic deletion of amino acids, strongly supports that the pronounced cleft is the enzymatic active site (6-8). Recently, the transition state analogues in structures of ricin and saporin ribosome-inactivating proteins were studied. The data confirmed that the invariant residues of RIPs in the catalytic active site of ricin were essential for the efficient catalysis by RTA (9). Although the biochemical properties of RIPs have been extensively studied, the enzymatic mechanism of RIPs is still elusive.

Deeply understanding of the catalytic mechanism of RIPs could help us develop potent neutralizing antibodies for protecting against ricin, a potential weapon of bioterrorism, and to design more effective therapeutic immunotoxins. Most of previous studies have demonstrated that antibody can be utilized as a powerful tool to investigate the structural and functional relationship of target protein (10,11). In the present study, we firstly employed antibodies obtained from individual mice immunized with RTA to study the relationship between the antibody recognition site on RTA and the neutralizing capacity of these RTA antibodies. In line with previous studies, we found that the antibodies specifically recognizing the enzymatic active site of RTA displayed substantial protective efficacy in vitro. One of the most striking findings in our present study is that the RTA monoclonal antibodies (mAbs) 6C2 and 6G3, whose combining site are distant from the catalytic active-site of ricin reported previously (5,7,12,13), exhibit more marked neutralizing ability than the Abs binding to the enzymatic active site of RTA. The computational and experimental data strongly indicated that the flexibility of an \(\alpha\)-helix, which is recognized by 6C2 and 6G3, plays an important role in regulating the enzymatic activity of ricin, suggesting that the \(\alpha\)-helix can serve as an attractive target for protection and rescue from ricin toxicity.

**EXPERIMENTAL PROCEDURES**

A detailed description of all of the procedures is provided in Supplementary Materials and Methods. Briefly, recombinant RTA was prokaryotically expressed in our laboratory. Then, RTA Abs were generated by immunizing Balb/c mice. Hybridomas secreting RTA mAbs were characterized by ELISA using isotope-specific reagents (Sigma) and purified from ascites fluid using protein A (GE Healthcare). The relative binding avidity of each mAbs was assessed by ELISA using RTA protein as the target antigen (14). The inhibition of the enzymatic activity of ricin by RTA mAbs was measured in a cell-free in vitro translation assay using rabbit reticulocyte lysates (Promega) as both the source of mRNA and ribosomes (14,15). Then, a standard MTT dye reduction assay was performed to evaluate the neutralization of ricin-mediated cytotoxicity. The in vivo mouse protection assays were done to evaluated the the in vivo protective efficacy of RTA mAb. The mice were randomized into several groups (n =
10 mice/group) and intraperitoneally injected with ricin holotoxin diluted in 0.2 ml of PBS (50 μg/kg). Subsequently, the mice were immediately administered with RTA mAbs (0.5 μg or 2.5 μg). For passive-transfer experiments, we administered ricin-challenged mice with a single dose of RTA mAbs at indicated time points (1 h, 2 h, 4 h or 8 h). The survival of mice was monitored until the experiment was terminated.

The RTA variants with different flexibility of the α-helix were designed based on inspection of the crystal structure of ricin. Then, the flexibility of the α-helix was evaluated by using molecular dynamic method. The dynamic simulations were performed with the AMBER 9.0 suite of programs (16). The detailed procedure can be seen under supplemental methods.

RESULTS

RIPs with very low similarity in primary structure are highly conserved in tertiary structure.

To date, the structures of fifteen type I RIPs and eight type II RIPs have been solved by X-ray crystallography (supplementary Table 1). Firstly, multiple protein sequences were aligned by using the Align123 algorithm, a progressive pairwise alignment algorithm modified from the CLUSTAL W program (17). Supplementary Figure 1 showed the best alignment of sequences of these twenty-three RIPs (fifteen type I RIPs and eight A chain of type II RIPs). Since the lengths of these proteins differ, some insertions or deletions were required for optimal alignment. Our data showed that the sequence identity among these RIPs was very low (2.3 %), and the sequence similarity was only 7.9 %. However, further study revealed that all of these type I RIPs and the A chain of the Type II RIPs have a very similar pattern in the secondary and tertiary structure (Figure 1A). When each is compared with RTA, the rms distance between corresponding Cα positions is generally less than 3.5 Å (supplementary Table 1). As we known, the solvent accessibility is much more related to the tertiary interactions between residues far apart in the sequence, but close in 3D space. The Figure 1B indicated that all of these RIPS exhibited a similar solvent accessibility. Moreover, the interesting feature in this alignment is the significant matching of hydrophobic amino acid residues in all twenty-three RIPS (supplementary Figure 1). Hydrophobicity plots were designed to display the distribution of hydrophobic and hydrophilic residues along a protein sequence and are useful for identifying both local and global properties for a protein sequence (18,19). In this study, the analysis of primary sequences of these RIPS by using a Kyte-Doolittle hydrophobicity plot indicated that these RIPS have a similar hydrophobic and hydrophilic pattern (Figure 1C). These data revealed that RIPS are highly conserved in the secondary and tertiary structures, although the similarity of the primary structures is very low. These findings may suggest that the evolutionary conserved tertiary structure may be essential for unique catalysis and rigid regulation of N-glycosidases.

Generation and characterization of monoclonal antibodies against ricin A chain

Due to exhibiting highly conserved tertiary structure with other RIPS and near perfect performance in depurination of the invariant adenosine residue from eukaryotic ribosome, ricin is an ideal prototype to understand the mechanism of toxicity of RIPS and to develop potent neutralizing antibodies for protecting against RIP toxins. Antibodies specific for different epitopes of RTA can be served as valuable tools for elucidating the structure-function relationship of RTA. Therefore, we firstly produced a panel of mAbs that bind determinants on the ricin A chain. Seventeen mAbs specific for RTA have been identified. As shown in Table S2, Ig subclass and
the protective effects of supernatant were evaluated. Among these anti-RTA mAbs, 6C2, 6G3, 13C10, 13G6 and 2D6 showed protective effects against toxicity of ricin from Vero cells (supplementary Table 2). An enzyme-linked immunosorbent assay was subsequently performed to measure the relative binding avidity of the RTA antibodies. As shown in Figure 2A, our data indicated that these five RTA mAbs had similar binding avidities. Then, these Abs were tested for their ability to inhibit ricin enzymatic activity, which was measured as the inhibition of protein synthesis in a cell-free system using rabbit reticulocyte lysates. In line with our results described above, 6C2, 6G3, 13C10 and 13G6 showed the best inhibition of ricin activity, with 2D6 exhibiting moderately inhibitory activity (Figure 2B). Next, the protective effects of these RTA mAbs were investigated by evaluating the viability of Vero cells after incubation with ricin (6.4 ng/ml) and the indicated concentrations of Abs (Figure 2C). In accordance with the protein inhibition experiment, the best neutralizing activity was seen with 6C2, 6G3, 13C10 and 13G6 (Figure 2B, C). To evaluate the correlation between in vitro neutralization and in vivo protection, we assessed the therapeutic activity of different neutralizing mAbs by in vivo mouse protection assay. Mice were injected intraperitoneally with ricin at a dose of 50μg/kg and immediately administered a single dose of mAbs. Notably, 0.5 μg of the neutralizing mAbs 6C2 and 6G3 provided potent protection efficacy (Figure 2D). In contrast, 13C10 and 13G6 only exhibited moderate protection against ricin. Our data indicated that even a single administration of 2.5μg 2D6 had slightly better protection than that of negative control (Figure 2E).

Since mAb 6C2 and 6G3 exhibited unusually strong protection against ricin both in vitro and in vivo, we further investigated the protective effects of these two mAbs by ascertaining the therapeutic window for post-exposure treatment using the BALB/c mouse model. As shown in Figure 2F, when ricin-challenged mice were administrated with 6C2 mAb at 2hr post-challenge, all animals were fully protected (100% survival). When 6C2 mAb treatment was delayed to 4hr, median survival in the 6C2 treated group was extended to 39.9hr, with statistically significant survival extension in this model by log-rank analysis (P < .05 compared with the untreated group). In line with these results, our data indicated that 6G3 showed a similar high level of protection against ricin in the post-exposure therapy (supplementary Figure 2). However, the protective effect of 13G6 could not be detected even though the mice were administered with 13G6 at 2hr post-challenge (Figure 2F).

Mapping of neutralizing monoclonal antibodies to RTA

To further investigate the reason of the variation in the neutralizing capacity of these anti-RTA mAbs, the immunological characterization of mAbs was carried out. As a preliminary form of epitope mapping, Antibody cross-inhibition assays among the anti-RTA mAbs were performed, in which the binding of an horseradish peroxidase-conjugated anti-RTA mAb was inhibited by unconjugated Abs (Figure 3A-B). Our data showed that RTA mAbs 6C2 and 6G3 appeared to have similar epitope specificity, which was inhibited by unconjugated 6C2 in a dose-dependent manner. Similarly, 13G6 and 13C10 had comparable patterns of inhibition, indicating that they blocked the binding of each other. Then, the epitopes of RTA mAbs were subsequently mapped with random peptide phage display libraries. 6C2 mAb binds to the amino acid sequence EXITH, which corresponds exactly to residues 102-106 of RTA. Phages selected by 13G6 mAb have a common motif of YYYSXXXT (supplementary Table 3). Overlaying this sequence on the three-dimensional structure of RTA (13,20) provides a best fit with residues 150-157 of RTA,
which fold into part of the enzyme active site (Figure 3C) (5). Mapping 2D6 mAb with random peptide library yielded a consensus sequence of IPXLPXVXR. The successfully characterized epitopes of these RTA mAbs were plotted onto the crystal structure of ricin (Figure 3C). It is interesting to find that, although the RTA mAb 6C2 and 6G3 display unusually strong neutralizing ability, the binding sites of these two mAbs are situated on an α-helix (residues 99-106 of RTA) far from the catalytic active site of ricin. However, the RTA mAb 2D6, whose binding site is located close to the enzymatic cleavage site of ricin, has very limited ability to protect against ricin toxicity both in vitro and in vivo.

The ideal recognition site for RTA neutralizing antibody is not located at the enzymatic active site of RTA

To investigate whether the significant protective effect of these RTA antibodies accounts for blocking of the interaction between RTA and substrate RNA, bicore binding studies were performed. We firstly synthesized the biotin-labeled stem-loop RNA (slRNA), which contains the required substrate motif for RTA. Then, RTA at the concentration of 60 μg/ml was allowed to bind to slRNA and a typical binding curve was obtained (supplementary Figure 3A). However, when we pre-incubated RTA with the excess amount (1:4 molar ratio) of 13G6, the binding signal can not be detected (supplementary Figure 3B). But the binding signal can be observed after incubation RTA with excessive amount of 6C2 or 2D6 (supplementary Figure 3C-D). We further investigate the binding affinity for the substrate with or without 6C2 and 6G3. The significant difference was not observed (Supplementary Table 4). Our data clearly demonstrated that the antibody 6C2 and 2D6 is unable to block the interaction between RTA and slRNA. But the 13G6 could completely block the binding of RTA to slRNA, when pre-incubated with RTA. Then, the extent of inhibition of RNA hydrolysis by RTA antibody was measured by HPLC determination of the amount of adenine released. As shown in supplementary Figure 3E, when we incubated excessive amount of mAb 6C2 or 13G6 with RTA, more than 80% of adenine released from ribosome could be inhibited. The noncompetitive inhibition constants (Ki) calculated for 6C2 and 6G3 are shown in supplementary Figure 3C-D. Our data indicated that 6C2 displays a similar inhibition constant to that of 6G3. The results revealed that 6C2 and 6G3 have a similar increase of neutralizing activity for ribosome. As shown in supplementary figure 4, 6C2 and 6G3 exhibited a similar dose-dependent inhibitory effect with 21%, 43% and 66% of inhibition at antibody concentrations of 0.033, 0.100 and 0.200 nM, respectively. All these results indicated that the substantial protective effect of 6C2 is not attributable to direct block of ricin-slRNA interaction, but disruption of depurination activity of RTA.

Rational design of RTA variants to investigate the functional role of the α-helix in regulating the enzymatic activity of ricin.

A considerable amount of research has studied the role of flexibility in enzyme action, indicating that the enzyme flexibility plays an important role in regulating the enzymatic activity (21,22). We suspect that the flexibility of the α-helix may play an important role in regulating the enzymatic activity of ricin. To investigate this issue, we rationally designed RTA variants with the different levels of the α-helix flexibility and evaluated the difference of these RTA variants in the depurination activity and protein synthesis inhibition. In the first, experimental variants were designed based on inspection of the crystal structure of ricin. As shown in Fig. 4A, Ala was substituted for Glu and Asp at position 99 and 100 to Ala was expected to disrupt the salt bridge formation with Arg48 and Arg56; Ala was substituted for
His and Leu at position 106 and 107 to avoid hydrophobic interactions with Ile53 and Phe57; Substitution of Asp96 was performed to prevent the formation of a weak salt bridge between Asp96 and Arg125. To investigate the role of the different point mutations we have introduced in RTA in modulating the flexibility of the α-helix (residues 99-106 of RTA), we carried out molecular dynamics simulations of RTA and its variants and analyzed the structural fluctuations throughout the MD trajectories.

The values of root mean square fluctuations (RMSF) reveal the extent of motion of an atom around its equilibrium position, providing information on flexibility or rigidity of molecules or parts of them (23,24). As shown in Figure 4B, the single point mutation D96A in RTA presented an increase in flexibility in the α-helix. When the temperature was elevated up to 500K, the mutation D96A or H106A/L107A could significantly increase the flexibility of the α-helix. But the double mutation E99A/D100A substantially reduced the flexibility of the α-helix even under the high temperature conditions (Figure 4B-C). The conformational change and dynamics of the α-helix were illustrated by showing root mean square deviation (RMSD) and by superimposing the coordinates of RTA and RTA variants. In line with the RMSF analysis, our data showed that a large fluctuation was produced by introducing the D96A mutation after 33 ns dynamic simulation (Figure 5A, C). The double mutation H106A/L107A could markedly increase the fluctuation of the α-helix under the high temperature, suggesting that the double mutation H106A/L107A has the potential to make the α-helix more flexible in room temperature. On the other hand, the double mutation E99A/D100A significantly decreased the flexibility of the α-helix even under the high temperature conditions (Figure 5B). Previous studies have demonstrated that there are four amino acids Y80, Y123, E177 and R180 critical for the depurination activity of ricin(6,9,12,25). To investigate how the flexibility of the α-helix affects the enzyme activity of ricin, we have performed dihedral angle analysis. Our results indicated that the flexibility of α-helix could affect the side-chain orientation of E177 (supplementary Figure 5).

After these RTA variants were successfully constructed and expressed, the amount of adenine released and subsequent inhibition of protein synthesis produced by RTA variants were studied. As shown in Figure 5D-E, introducing the point mutation at position 96 (RTA_D96A) or at position 106 and 107 (RTA_H106A/L107A) led to a substantial increase in the toxicity of RTA, but the double mutation RTA_E99A/D100A can substantially decrease the amount of adenine released and protein synthesis inhibition. In line with our expectations of the rational design, these data clearly demonstrate that the flexibility of the α-helix is responsible for modulating the depurination activity of RTA and ultimately the degree of inhibition of protein synthesis.

DISCUSSION

In our present study, we firstly collected a set of PDB structures of RIPs from Protein Data Bank (PDB). Then, bioinformatic methods for analyzing the sequence-structure relationship of RIPs have been performed. It is striking to note that, although all these RIPs have a very low sequence similarity, the secondary and tertiary structures are highly conserved. Many previous investigations have revealed that RIPs depurinate RNA in ribosomes at a specific universally conserved position in the rat 28S rRNA(1-3), suggesting that the highly conserved tertiary structure may be responsible for unique catalytic properties of RIPs. Further analysis indicated that all these RIPs had a similar pattern in the distribution of hydrophobic and hydrophilic residues along the protein sequence.
Previous studies have collectively demonstrated that the hydrophobic effect is the dominant force in protein folding and protein stability (26-29). In agreement with these results, our data suggest that the hydrophobic distribution pattern might play a significant role in determining the secondary and tertiary structures of RIPs, which are highly conserved in ribosome depurination.

Previous studies have demonstrated that the catalysis of 80S ribosome by RTA approached the diffusion rate limit for enzymatic reactions with a catalytic efficiency of $2.6 \times 10^8$ M$^{-1}$s$^{-1}$ for the discontinuous assay conditions (15,30). Among these RIPs, ricin has evolved to become a near-perfect catalyst for mammalian ribosomes. It can be seen as an ideal prototype to investigate the N-glycosidase mechanism of RIPs. In addition, epitope-specific mAbs have been widely used as a powerful tool to clarify the relation between the structure and the function of macromolecule (31,32). Therefore, we generated a panel of mAbs raised against ricin A-chain, the catalytic subunit of ricin, to study the structure-function relationship of ricin. Both in vitro and in vivo assays were performed to evaluate the neutralizing ability of RTA mAbs. Although 6C2 and 13G6 have a similar protective efficacy in our in vitro experiments, our in vivo protection data clearly showed that, the mAb 6C2 and 6G3, whose binding site are far from the enzymatic active site, have more potent neutralizing activity against ricin than the RTA mAb 13G6 recognizing the catalytic active site of ricin. Our biacore data revealed that the exceptional neutralization potency of 6C2 is not attributed to the block of ricin-siRNA interaction, but the direct inhibition of depurination activity of ricin. Due to the combining site of mAb 6C2 and 6G3, whose binding site are far from the enzymatic active site of ricin, the ricin-antibody complex formation can not be blocked by the substrate. Thus, the mAb 6C2 and 6G3 could continuously play a functional role in inhibition of depurination activity of ricin in vivo, which may be the reason why 6C2 and 6G3 exhibited more potent in vivo protective efficacy than 13G6.

The data also indicate that the $\alpha$-helix recognized by 6C2 may play a significant role in controlling the depurination activity of ricin. To further investigate the role of the $\alpha$-helix in regulating the enzymatic activity of ricin, molecular dynamic method was employed to rationally design RTA variants with difference in the flexibility of the $\alpha$-helix. Our subsequent experimental data clearly revealed that increasing the flexibility of the $\alpha$-helix could substantial enhance the depurination activity of ricin and the inhibition of protein synthesis. On the other hand, the marked decrease in the flexibility of the $\alpha$-helix has significant reduced the enzymatic activity of ricin. As we known, binding by antibody could substantial decrease the flexibility of the targeted region. Thus, binding by 6C2 could substantial reduce the flexibility of the $\alpha$-helix and substantially decrease the depurination activity of ricin. These data suggested that the flexibility of the $\alpha$-helix play a critical role in regulating the toxicity of ricin. Furthermore, we have performed dihedral angle analysis to study the relationship of the $\alpha$-helix and catalytic active site of ricin. We found that the flexibility of the $\alpha$-helix could affect the side-chain orientation of Glu177 (supplementary Figure 5). Previous studies have identified that E177 is critical for the depurination activity of ricin (5,6,9,12). Taken together, these data suggested that the $\alpha$-helix may modulate the enzymatic activity of ricin by controlling the side-chain orientation of Glu177.

In summary, our data clearly demonstrate that the $\alpha$-helix recognized by 6C2 is responsible for modulation of ricin toxicity, suggesting that the $\alpha$-helix might be used as an ideal target for the prevention and treatment of ricin toxicosis. Additionally, our bioinformatic analysis revealed that RIPs are highly conserved in the secondary and tertiary structures and the conserved $\alpha$-helix may be critical for the depurination activity of
other RIPs.

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**FOOTNOTES**

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The abbreviations used are: RIPs, ribosome-inactivating proteins; RTA, ricin A-chain; mAbs, monoclonal antibodies; siRNA, stem-loop RNA; RMSF, root mean square fluctuations; RMSD, root mean square deviation; PDB, Protein Data Bank.
FIGURE LEGENDS

Figure 1. Structure analysis of RIPs. Stereoview of the superimposed backbone atoms for the structures of RIPs. (A) Secondary structure elements are colour coded as: red – helix; cyan – strand; white – coil. (B) Hydrophobic and hydrophilic residues are colored green and blue. (C) Kyte-Doolittle hydropathy analysis using a window of five amino acids. Hydrophobicity is shown as the vertical axis with the hydrophobic side of the plot having a positive value. The horizontal axis shows the amino acid residue number along the sequence.

Figure 2. Characterization and evaluation of mAbs against RTA. (A) Relative binding avidity of RTA mAbs. RTA mAbs were purified on protein G. Purified mAbs were titrated in Enzyme-linked immunosorbent assay (ELISA) for binding to RTA (100 ng/well). (B) Inhibition of ricin enzymatic activities by RTA mAbs. A fixed concentration of ricin holotoxin was mixed with varying concentrations of RTA mAb. The ability of mAbs to block the inhibition of cell-free protein synthesis was measured. (C) Ab-mediated inhibition of ricin cytotoxicity. Vero cells were incubated with ricin (1μg) and the indicated Ab. After 40 hrs, cell viability was assayed by MTT dye reduction. (D, E, F) Therapeutic efficacy of RTA mAbs. After an intraperitoneal injection of ricin into Left-sidedness on the basis of weight (50μg/kg), mice was administrated with a single dose of 0.5 μg (D) or 2.5 μg (E) of RTA mAb. Data reflect approximately 10 mice per condition. (F) Efficacy of RTA-specific mAb therapy at different time points. A single dose (5μg) of mAb 6C2 was administered either 1h, 2h, 4h or 8h after Ricin injection. Data are mean ± SD of at least 3 experiments.

Figure 3. Epitope Identification for RTA mAbs. (A, B) Microtiter plates were coated with 50 ng/well of RTA. Unconjugated RTA mAbs (5μg/ml), indicated on the horizontal axis, were added to the coated microtiter wells and incubated for 60 min, the horseradish peroxidase (HRP)-conjugated Ab indicated at the top of the graph was added, and the mixture was incubated for 60 min. The plates were washed, and colorimetric substrate was added. Antibodies were tested in up to four different experiments, of which this is representative. (C) Epitope mapping of RTA mAbs. Epitopes defined by mapping using peptide display phage libraries were plotted onto the three-dimensional structure of RTA. Red indicates the epitope of RTA mAb 6C2 and 6G3, green the epitope of mAb 2D6, yellow enzyme active site, and blue residue present in the active site and bound by RTA mAb 13G6 and 13C10. The graphs are representative of at least 3 experiments, each of which showed similar results.

Figure 4. Rational design of RTA variants. (A) Stereodiagram showing the overall fold of the RTA. The epitope of mAb 6C2 is colored in blue. (B, C) The average fluctuation per residue calculated over the last 40 ns trajectory. Root mean square fluctuation (RMSF) values were calculated relative to the starting structure at 300 K (B) and 500K (C).

Figure 5. Characterization and comparison of RTA variants. (A, B) RMS deviations for the α-helix (residues 99-106 of RTA). Molecular dynamic simulation of RTA variants were...
performed at 300K (A) and 500K (B). The last 40 ns of each trajectory was used for analysis.

(C) The average structures of each RTA variant over the last 10 ns of simulation at 300K are superimposed and shown. RTA is colored in gray, RTA_D96A is in red, RTA_E99A/D100A is highlighted in blue and RTA_H106A/L107A is colored in green. (D) Quantity of adenine released from 80S rabbit reticulocyte ribosomes. The extent of ribosome depurination was quantified by using HPLC. (E) The extent of protein synthesis inhibition by RTA variants was evaluated in cell-free assays. The different concentrations of RTA variants were incubated with a cell-free in vitro translation mixture. Luciferase activity reported in relative light intensity, is representative of protein translation and measured after 90min at 30 °C. Data are mean ± SD of at least 3 experiments.
Fig. 3

A

6C2-HRP

OD450

0 50 5 0.5 0.05 Ab concentration (ug/ml)

1.2

0.8

0.4

0.2

0.0

0.2 8 0.8 0.08 Ab concentration (ug/ml)

0.2 8 0.8 0.08 Ab concentration (ug/ml)

B

13G6-HRP

OD450

0 80 8 0.8 0.08 Ab concentration (ug/ml)

0.2 8 0.8 0.08 Ab concentration (ug/ml)

C

mAb 6C2 and 6G3

mAb 2D6

catalytic active site

mAb 13G6 and 13C10
Fig. 4

A  α-helix

B 300K

C 500K
Fig. 5

A

300K

RMSD vs Time (ns)

B

500K

RMSD vs Time (ns)

C

D

E

Adenine released (pmol)

Light intensity (AU)

Protein Concentration (µg)

RTA, RTA D96A, RTA E99A/D100A, RTA H106A/L107A

1E-3 0.01 0.1
Identification of a novel functional domain of ricin responsible for its potent toxicity

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