Cytotoxicity of a recombinant ricin-A-chain fusion protein containing a proteolytically-cleavable spacer sequence

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Chimeric proteins composed of ricin toxin A chain (RTA) and staphylococcal protein A (PA) have been produced in *E. coli*. Constructs consisting of N-terminal RTA and C-terminal PA (RTA-PA) or N-terminal PA and C-terminal (PA-RTA) were capable of binding to immunoglobulin G (via PA) and of specifically depurinating 28 S ribosomal RNA (via RTA). However, neither fusion protein was cytotoxic to antigen-bearing target cells in the presence of an appropriate monoclonal antibody presumably because the RTA could not be released from the PA within the cytosol where the ribosomal substrate of RTA is located. The overcome this, a short amino acid sequence from diphtheria toxin was engineered between the RTA and PA to produce a disulfide-linked loop containing a trypsin sensitive cleavage site. Cleavage of this fusion protein with trypsin converted the RTA-DT-PA to the two chain form consisting of RTA linked by a disulfide bond to PA. The cleaved fusion protein was highly toxic to Daudi cells coated with anti-immunoglobulin antibody suggesting that the RTA could be released from the PA by reduction within the cytosol.

Ricin A chain; Fusion protein; Protein A; Proteolytically-cleavable sequence

1. INTRODUCTION

The A chain of the potent cytotoxin ricin inhibits eukaryotic protein synthesis by catalytically inactivating the 60 S ribosomal subunit [1]. This results from the *N*-glycosidic cleavage of a specific adenine residue from a highly conserved stem-loop structure of 28 S ribosomal RNA [2,3]. Depurination at this site renders the 28 S rRNA unable to bind elongation factors [4]. In the holotoxin, the catalytic A chain is joined to a galactose-binding B chain by a single disulfide bond [1]. Cytotoxicity is mediated by the B chain which interacts with galactose residues of glycoproteins and glycolipids on the surface of target cells, and which is believed to play a role in transporting the A chain into the cytosol [1,5,6]. Ricin A chain (RTA) is only catalytically active after release from the B chain (RTB) by reductive cleavage of the disulfide bond [7].

Bacterial toxins such as diptheria toxin or *Pseudomonas* exotoxin A contain 3 functionally distinct domains. These domains are responsible for target cell binding, translocation of the toxin across intercellular membranes and ADP-ribosylation of elongation factor 2 (the step by which these bacterial toxins cause cell death), respectively [8,9]. Recombinant fusion proteins comprising fragments of these toxins and cell-binding ligands have been produced by expressing chimeric genes in *Escherichia coli*. The native cell binding domain of the toxin has been replaced with, for example, interleukin-2 [10], α-melanocyte stimulating hormone [11], transforming growth factor type α [12] or antigen-binding antibody fragments [13]. The resulting fusion proteins exert potent and specific cytotoxic effects upon target cells bearing the appropriate receptor or antigen.

Similar types of fusion proteins containing plant toxins such as RTA have not been described. In ricin-based immunotoxins, the RTA is normally linked to an antibody via a disulfide bond [14]: RTA immunotoxins constructed with linkages lacking disulfide bonds have much weaker cytotoxic activity, probably because release of the RTA by reduction in the cytosol is required for cytotoxicity. In the present report, we described the production of recombinant fusions between RTA and staphylococcal protein A (PA). While both components of the fusions are biologically active, the chimeric proteins are not cytotoxic to antibody-coated cells. Cytotoxicity can be conferred, however, by joining the two components of the fusion via a disulfide-linked peptide loop containing a trypsin-sensitive cleavage site.

2. MATERIALS AND METHODS

2.1. *Expression plasmids*

All plasmids were derivatives of pRIT5 (Pharmacia) which contain the *S. aureus* PA coding sequence, including the 5′ signal sequence, downstream from the PA promoter. Plasmid pPA-RTA was produc-

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ed by ligating an 879 bp *Bam*H1 fragment derived from pRIC [15], which contained the entire RTA coding sequence, in frame into the *Bam*H1 site of pRIT5. Plasmid pRTA-PA was produced by ligating a 836 bp end-filled *Xho*1-*Kpn*1 fragment, which contained the entire RTA coding sequence, into a unique, end-filled *Bcl*1 site in pRIT5. This results in the insertion of the RTA sequence into the PA sequence within the region encoding the first of the 5 antibody binding domains. The 5′ region of the 836 bp *Xho*1-*Kpn*1 fragment was derived from the RTA M13 clone mutagenized to create the *Xho*1 site at position −15 [15], and the 3′ region was derived from the original preproricin cDNA [16]. A translational termination signal (contained in the Omega fragment [17]) was introduced after the PA coding sequence in the *Eco*RI site of the pRIT5 multiple cloning region.

The construction of plasmid pRTA-DT-PA involved 3 steps. Firstly, the omega region of pHP45Ω [17] was isolated as a 2 kbp *Bam*H1 fragment and digested into two halves using *Bcl*1. A single half-omega fragment was isolated and ligated into the pRIT5 multiple cloning region which had been digested with *Eco*RI, end filled, and redigested with *Bam*H1. The resulting construct provides translational stop codons in all reading frames after the PA coding sequence. The vector was further modified by deleting the *Pst*1 and *Sal*1 multiple cloning sites. Secondly, a synthetic oligonucleotide containing a series of restriction sites (*Eco*RI, *Xho*1, *Bam*H1, *Bgl*11 and *Pst*1) was ligated into the unique *Bcl*1 site at position 66 in the PA coding sequence of pRIT5 generating pRIT5Ω. Thirdly, a 90-mer oligonucleotide encoding the diphtheria toxin (DT) trypsin sensitive site was ligated as a *Bam*H1-*Pst*1 fragment (Fig. 1a) into the new *Bam*H1 and *Pst*1 sites of pRIT5Ω. Encoded within this oligonucleotide are two cysteines which form a disulfide bond in DT and which, after normal proteolytic nicking of the bacterial toxin, covalently link the A and B fragments together [18]. Finally, the DNA encoding RTA was provided as a 867 bp *Xho*1-*Bam*H1 fragment and ligated into the new *Xho*1 and *Bam*H1 sites of pRIT5Ω. The final construct encodes a 631 residue protein fusion comprising a 36 residue signal peptide, 22 residue of PA, 288 residues of RTA and linker, 34 residues of multiple cloning site remnants and DT loop and 251 residues of protein A. The fusion proteins encoded by the 3 expression vectors are illustrated schematicaly in Fig. 1b. For expression, the recombinant plasmids were introduced into *E. coli* strain 71.18 by $CaCl_2$-mediated transformtion. Cultures (500 ml) were grown at 30°C in the presence of ampicillin (500 ml) to early stationary phase (A_{550} = 1.0). *E. coli* cells were harvested by centrifugation and resuspended in 10 ml of 10 mM Tris-HCl, pH 7.4, 20% sucrose. The periplasmic fraction was isolated by osmotic shock as described [19].

2.2. *Isolation of recombinant proteins*

The periplasmic fraction was applied to an IgG-Sepharose 6FF column (1 × 6 cm, Pharmacia) that had been equilibrated in PBST (0.15 M NaCl, 10 mM Na phosphate, pH 7.4, contasining 0.05% Tween 20). The column was washed with PBST until no protein was detected in the flow-through, and the bound material was then eluted with 0.1 M glycine-HCl, pH 3.0. The eluted sample was neutralized immediately with 3 M Tris-HCl buffer pH 8.0 and dialysed overnight against PBS.

2.3. *Inhibition of protein synthesis*

The catalytic activity of RTA in the recombinant fusion proteins was determined by their ability to inhibit [^{14}C]leucine incorporation into protein in a rabbit reticulocyte lysate [20]. A range of concentrations (0.1–1000 ng/ml) of recombinant proteins or purified native RTA were assayed for inhibition with respect to controls over a 5 min translational period at 28°C. Each concentration was assayed in triplicate. Evidence for RTA depurination of 28 S rRNA was obtained by aniline hydrolysis of total RNA extracted from the translational mixtures as described [21].

2.4. *Trypsin cleavage of the DT loop*

Recombinant RTA-DT-PA was incubated with trypsin (1 μg/ml in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) at 25°C for 30 s. Digestion was stopped by adding soybean trypsin inhibitor (5 μg/ml in 50 mM Tris-HCl, pH 8.0).

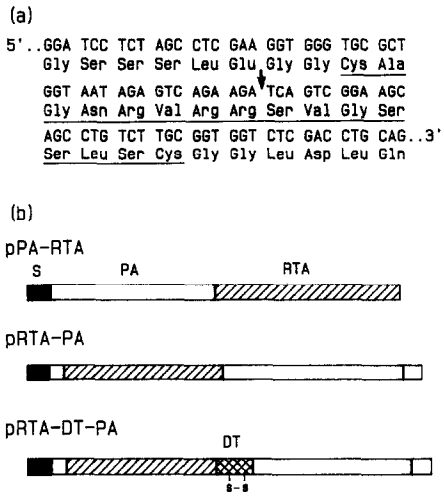

Fig. 1. (a) Nucleotide and amino acid sequence of a 90-mer *Bam*H1-*Pst*1 fragment, which includes the trypsin cleavage site (arrowed) of the DT disulfide loop (underlined), and (b) schematic illustration of the fusion proteins encoded by the expression plasmids generated.

2.5. *Ricin B-chain (RTB)-mediated toxicity*

RTB-mediated cytotoxicity of RTA-DT-PA was measured by incubating RTA-DT-PA, without or after trypsin treatment, with RTB (Inland Laboratories, Dallas, TX) for 2 h at 8–10°C followed by 2 h at room temperature. Test mixtures were added in 25 μl volumes in phosphate buffered saline (PBS) to PBS-washed Vero cells, dispensed into 96 well microplates at 2 × 10^4 cells per well. After 1 h at 37°C, 100 μl/well of DMEM was added and the plates were incubated for a further 20–24 h. Each well was then pulsed for 2 h with 1 μCi [^{35}S]methionine in 50 μl PBS, 0.5 M NaOH was added and the radioactivity was determined by scintillation counting.

2.6. *Cytotoxicity of RAT-PA to antibody-coated cells*

RTA-PA, PA-RTA and RTA-DT-PA fusions which had been treated or not treated with trypsin as above were mixed with equimolar quantities of rabbit anti-human Kappa light chain antibody (Sigma, Poole, UK). The mixtures were stored for 1 h at 4°C. Daudi lymphoblastoid cells, which express surface immunoglobulin (K light chain), were suspended at 4 × 10^5 cells/ml in RPMI 1640 Medium containing 0.5% w/v bovine serum albumin (BSA), insulin, transferrin and selenite supplement (Sigma), 200 U/ml penicillin and 100 μg/ml streptomycin (serum-free medium). The cells were distributed in 100 μl volumes into the wells of 96-well flat-bottomed microtitre plates and 100 μl of the test materials in serum-free medium was added. The plates were incubated for 24 h at 37°C in an atmosphere of 5% CO_2 in humidified air. After this time, 20 μl of foetal calf serum and 1 μCi [^{3}H]leucine (Amersham Radiochemicals Ltd, UK) was added to each culture. The cultures were incubated for a further 24 h at 37°C. The cells were then harvested onto glass fibre discs using a Skatron automated cell harvester. The radioactivity on the disc was measured using a Packard Rac-beta liquid scintillation counter. The [^{3}H]leucine incorporation of treated cells was compared with that of untreated cells to obtain the % inhibition of protein synthesis resulting from the treatment. In an alternative assay trypsin-treated or untreated RTA-DT-PA was mixed with WT1 (a mouse monoclonal anti-CD7 IgG2a) or with HRS-1 (a mouse monoclonal IgG2a antibody of irrelevant specificity) before being incubated with CD7$^+$ Jurkat cells which were processed as described above. The WT1 antibody was a gift from Dr W. Tax, Nijmegen, The Netherlands, and the HRS-1 antibody was a gift from Dr M. Pfrenndschuk, Cologne, FRG.

3. RESULTS AND DISCUSSION

3.1. *Expression and purification of fusion protein*

E. coli cells transformed with each of the expression plasmids prepared here produced soluble, recombinant products of the expected molecular weight which were not formed in control, non-transformed cells. The PA component of the fusion proteins was functionally active, following the products to be purified from the periplasmic fraction in a single step by affinity chromatography on IgG-Sepharose (Fig. 2).

Identification of the purified products was confirmed by Western blotting: all 3 fusion proteins crossreacted strongly with rabbit antibodies raised against recombinant ricin A chain [15]. The purified products frequently appeared as a doublet on gels (Fig. 2). We assume that the less mobile component has not had the PA signal sequence proteolytically removed while the more mobile one has. We have previously noted that the extent to which a bacterial signal sequence preceding a recombinant protein targeted to the periplasmic space of *E. coli* is processed is host strain dependent [19].

3.2. *Ribosome inactivation by the fusion proteins*

The purified fusion proteins were potent inhibitors of protein synthesis in rabbit reticulocyte lysates. RTA-PA and RTA-DT-PA had an IC_{50} (the concentration required for 50% inhibition of protein synthesis) of around 30 ng/ml, and were slightly more effective than PA-RTA (IC_{50} 90 ng/ml), but were an order of magnitude less active than purified RTA (IC_{50} 3 ng/ml). The observed inhibition of protein synthesis resulted from the catalytic activity of the RTA component on its rRNA substrate. RTA depurinates 28 S rRNA at a specific site close to the 3′ end of the molecule. Depurination renders isolated rRNA susceptible to amine-catalysed hydrolysis of the phosphodiester bonds on either side of the modified site. This cleavage generated a small RNA fragment of about 390 ribonucleotides from reticulocyte 28 S rRNA which is therefore diagnostic of RTA ribosome inactivation. Reticulocyte ribosomes incubated with RTA-PA, PA-RTA and RTA-DT-PA all released the characteristic RNA fragment upon aniline treatment of the isolated rRNA (Fig. 3).

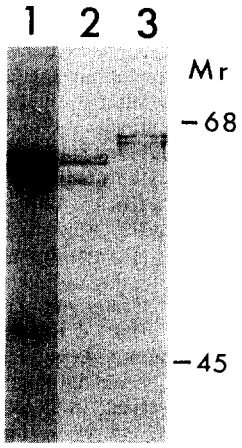

Fig. 2. Affinity purification of recombinant fusion proteins. The periplasmic fraction from *E. coli* expressing PA-RTA (lane 1), RTA-PA (lane 2) and RTA-DT-PA (lane 3) were passed down an IgG-Sepharose column. Bound material was eluted, separated by Na $DodSO_4$/PAGE and stained with Coomassie blue.

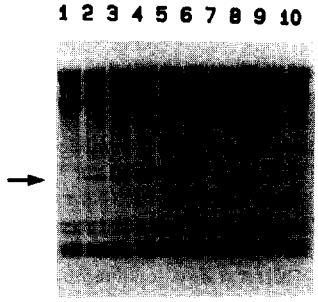

Fig. 3. Depurination of 28 S rRNA. Control ribosomes (lanes 1 and 6) or ribosomes incubated with purified RTA (lines 2 and 7), PA-RTA (lanes 3 and 8), RTA-PA (lanes 4 and 8) and RTA-DT-PA (lanes 5 and 10) were treated with aniline (lane 1 to 5) or untreated (lanes 6 to 10), and the RNA was extracted and fractionated. The arrow indicates the 390 ribonucleotide fragment released from RTA-modified ribosomes.

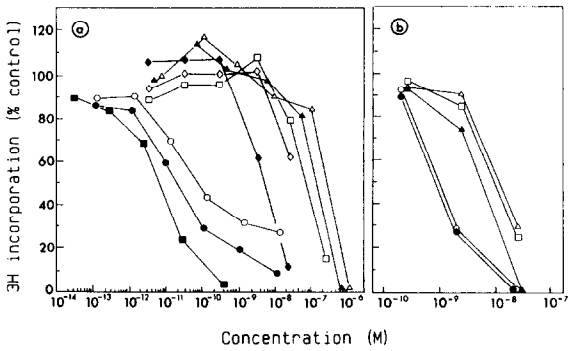

Fig. 4. (a) Inhibition of protein synthesis in Daudi cells. A range of concentrations of purified recombinant fusion proteins were mixed with equimolar quantities of rabbit anti-human Kappa light chain antibody and incubated with Daudi cells as described in the Materials and Methods before determining the ability of the cells to incorporate [^{14}C]leucine into protein. Cells were incubated with RTA-PA (▲), PA-RTA (△), RTA-DT-PA without trypsin cleavage (○), RTA-DT-PA after trypsin cleavage (●). In other cultures cells were incubated with RTA (□), whole ricin (■), RTA-DT-PA without trypsin cleavage (◇) and RTA-DT-PA after trypsin cleavage (◆), all of which were not preincubated with rabbit anti-human Kappa light chain. (b) Inhibition of protein synthesis in Jurkat cells. RTA-DT-PA was mixed with equimolar quantities of mouse anti-CD7 or with control mouse IgG2a antibody and incubated with Jurkat cells: RTA-DT-PA with anti-CD7 (○) or with control antibody (△) without trypsin cleavage; RTA-DT-PA with anti-CD7 (●) or with control antibody (▲) after trypsin cleavage. In other cultures, cells were incubated with RTA alone (□). Points represent the arithmetic means of triplicate determinations, the standard deviation of which were all less than ± 10% of the mean values as plotted.

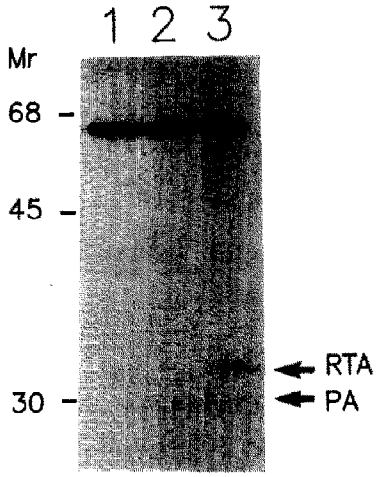

Fig. 5. Electrophoretic analysis of RTA-DA-PA before (lane 1) or after (lane 2 and 3) brief trypsin treatment in the presence (lanes 1 and 3) or the absence (lane 2) of dithiothreitol. The arrows indicate the positions of the release RTA and PA bands.

3.3. *Cytotoxicity*

Recombinant RTA-PA and PA-RTA premixed with rabbit anti-human Kappa light chain (RAHk) were not cytotoxic to Daudi cells. Their IC_{50} values were greater than that of free RTA (10^{-7} M or greater) (Fig. 4a). In contrast, RTA-DT-PA premixed with RAHk was cytotoxic to Daudi cells ($IC_{50} = 8\times10^{-11}$ M). Trypsin treatment of RTA-DT-PA increased its toxicity approximately 4-fold ($IC_{50} = 2\times10^{-11}$ M). Thus inclusion of the DT sequence and cleavage site in the recombinant fusion protein significantly enhances cytotoxicity. In the presence of antibody, the IC_{50} for the trypsin-treated chimeric protein approached that of whole ricin (6×10^{-12} M (Fig. 4a)). The toxicity of RTA-DT-PA premixed with RAHk for Daudi cells was specific since RTA-DT-PA applied alone was several 100-fold less toxic ($IC_{50} = 3\times10^{-8}$ M for non-cleaved RTA-DT-PA and 6×10^{-9} M for cleaved RTA-DT-PA (Fig. 4a)) and RTA-DT-PA mixed with a mouse IgG2a antibody (which was also able to bind PA) was no more cytotoxic than RTA-DT-PA added alone (data not shown).

In an alternative cytoxicity assay RTA-DT-PA premixed with mouse anti-CD7 antibody specifically killed $CD7^+$ Jurkat cells (Fig. 4b), although the potency was low ($IC_{50} = 10^{-9}$ M) and it was only 10-fold higher than that observed with a control mouse IgG2a antibody of irrelevant specificity (Fig. 4b). Trypsin cleavage did not increase the potency of RTA-DT-PA in the presence of anti-CD7 antibody suggesting, as did the experiment shown in Fig. 4a, that cellular proteolytic cleavage of RTA-DT-PA was occurring.

The DT loop was specifically susceptible to trypsin digestion. After digestion, the treated product ran with an apparent molecular weight indistinguishable from undigested product when electrophoresed under non-reducing conditions (Fig. 5, lanes 1 and 2). Under reducing conditions, two smaller bands were observed in the digested sample (Fig. 5, lane 3, arrowed) which were the expected apparent size for the RTA and PA components of the fusion protein. The bands released by limited trypsin digestion were difficult to visualize, but more prolonged trypsin treatment resulted in proteolysis of the released RTA and PA polypeptides. The RTA band was positively identified by Western blotting using anti-recombinant RTA antibodies, which also bound to a second band whose apparent size was that expected for PA (data not shown).

Further evidence that proteolytic cleavage of RTA-DT-PA liberated a potentially toxic RTA fragment was obtained using RTB-mediated cytotoxicity assays in which RTB reassociated with the liberated RTA to form a ricin-like adduct. Recombinant RTA-DT-PA was not toxic to Vero cells, even in the presence of 10^{-8} M purified RTB (TAble I). After trypsin treatment, however, RTA-DT-PA became potently cytotoxic in the presence of RTB (Table I).

The fusion proteins RTA-PA and PA-RTA were not cytotoxic to target cells in the presence of an appropriate cell-reactive antibody. This accorded with the finding with chemically-linked antibody-RTA conjugates that a disulfide linkage between the two protein components is necessary for cytotoxicity [22]. This suggested that the lack of toxicity of the RTA-PA and PA-

Table I

Effects of trypsin treatment of RTA-DT-PA on RTB-mediated inhibition of protein synthesis in Vero cells

Addition	Trypsin treatment	(% control)
None (control)	No	100
10^{-8} M RTB	No	111
1.5×10^{-7} M RTA-DT-PA	No	102
1.5×10^{-7} M RTA-DT-PA + 10^{-8} M RTB	No	98
1.5×10^{-9} M RTA-DT-PA + 10^{-8} M RTB	Yes	84
1.5×10^{-8} M RTA-DT-PA + 10^{-6} M RTB	Yes	24
1.5×10^{-7} M RTA-DT-PA + 10^{-8} M RTB	Yes	8

RTA-DT-PA, with or without trypsin treatment, was mixed with RTB before incubation with Vero cells. The ability of the cells to incorporate [^{35}S]methionine into protein was determined. Each assay was performed in triplicate. 10^{-8} M RTA plus 10^{-8} M RTA gave 98% inhibition of protein synthesis.

RTA was because the RTA could not be released from the PA by reduction within the cytosol in order to inactivate the cells' ribosomes. We overcame this problem by inserting a cleavable linker, the DT loop, between the RTA and PA moieties. Trypsin cleavage of this linker resulted in RTB-mediated cytotoxicity to Vero cells (Table I) and antibody-mediated cytotoxicity to cells (Fig. 4). In the case of antibody-mediated cytotoxicity, prior cleavage with trypsin was not an absolute requirement; presumably proteases within the target cell can cleave the DT loop in the construct, as they do with non-nicked diphtheria toxin itself [8].

In an earlier study by Weaver et al. [23], functional recombinant PA-RTA was shown to be capable of binding specifically to antigen-bearing target cells. There was, however, no indication that the fusion protein was cytotoxic. By contrast, disulfide-linked RTA and PA prepared by chemical means has been shown to be potently cytotoxic to antigen-bearing cells [24,25]. The present study demonstrates that recombinant RTA-PA fusions can also be cytotoxic provided that they contain an appropriate internal cleavage site which allows the generation of free RTA within the target cell.

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