Inclusion of a furin-sensitive spacer enhances the cytotoxicity of ribotoxin restrictocin containing recombinant single-chain immunotoxins

Anita GOYAL and Janendra K. BATRA[1]

[1]Immunochemistry Laboratory, National Institute of Immunology, Aruna Asaf Ali Road, New Delhi-110067, India

Chimaeric toxins have considerable therapeutic potential to treat various malignancies. We have previously used the fungal ribonucleolytic toxin restrictocin to make chimaeric toxins in which the ligand was fused at either the N-terminus or the C-terminus of the toxin. Chimaeric toxins containing ligand at the C-terminus of restrictocin were shown to be more active than those having ligand at the N-terminus of the toxin. Here we describe the further engineering of restrictocin-based chimaeric toxins, anti-TFR(scFv)–restrictocin and restrictocin–anti-TFR(scFv), containing restrictocin and a single chain fragment variable (scFv) of a monoclonal antibody directed at the human transferrin receptor (TFR), to enhance their cell-killing activity. To promote the independent folding of the two proteins in the chimaeric toxin, a linear flexible peptide, Gly-Gly-Gly-Gly-Ser, was inserted between the toxin and the ligand to generate restrictocin–linker–anti-TFR(scFv) and anti-TFR(scFv)–linker–restrictocin. A 12-residue spacer, Thr-Arg-His-Arg-Gln-Pro-Arg-Gly-Trp-Glu-Gln-Leu, containing the recognition site for the protease furin, was incorporated between the toxin and the ligand to generate restrictocin–spacer–anti-TFR(scFv) and anti-TFR(scFv)–spacer–restrictocin. The incorporation of the proteolytically cleavable spacer enhanced the cell-killing activity of both constructs by 2–30-fold depending on the target cell line. However, the introduction of linker improved the cytotoxic activity only for anti-TFR(scFv)–linker–restrictocin. The proteolytically cleavable spacer-containing chimaeric toxins had similar cytotoxic activities irrespective of the location of the ligand on the toxin and they were found to release the restrictocin fragment efficiently on proteolysis *in vitro*.

Key words: chimaeric toxins, protease, protein engineering, targeting, toxins.

INTRODUCTION

The lethal potential of plant and bacterial toxins has been used extensively to generate immunotoxins and chimaeric toxins for targeted therapy [1–5]. Immunotoxins and chimaeric toxins consist of potent protein toxins linked to targeting ligands by chemical coupling or gene fusion technology. The bacterial toxins *Pseudomonas* exotoxin A (PE) and diphtheria toxin (DT) have been used successfully for making active immunotoxins and chimaeric toxins, which have undergone preclinical and clinical trials [2,6]. Immunotoxins containing the plant toxin ricin have also shown great promise in clinical trials; however, the generation of plant recombinant immunotoxins is lagging behind [1]. Because recombinant immunotoxins are made by gene fusion technology, certain constraints are inherent in their constructions in terms of the orientation of ligand and the toxin in the fusion protein. With PE, a ligand has to be attached at the N-terminus, whereas a free N-terminus is absolutely essential for the cytotoxicity of DT and ligands have to be placed at its C-terminus [7,8]. Chimaeric toxins bind specifically to the cell-surface target; after internalization, the toxin is translocated to the intracellular target to manifest its cytotoxic activity via different routes for different toxins [1,6]. The PE and DT containing chimaeric toxins are cleaved proteolytically inside the cell; a translocation-competent and enzymically active fragment of toxin thus released acts on its intracellular target [9–11]. The ricin-A-chain-based chimaeric toxins with direct gene fusions of ligand and toxin were found to be functionally inactive; however, the insertion of

an intervening specific protease recognition site made them cytotoxic [12,13]. Chimaeric toxins are being explored as alternative modality for the treatment of diseases such as cancers, HIV infection, autoimmune disorders and various neural disorders [1,4,5,14]. The results indicate therapeutic efficacy; however, dose-dependent systemic toxicity and immunogenicity are now being recognized as the major hurdles in their exclusive use [2,3,15]. There is therefore always a need to explore novel toxins with desirable properties that could be readily used as components of chimaeric toxins with any potential targeting ligand.

Restrictocin, a specific ribonuclease produced by the fungus *Aspergillus restrictus*, belongs to a family of ribosome-inactivating proteins classified as ribotoxins. α-Sarcin and mitogillin are two other well-characterized members of the ribotoxin family [16]. These proteins share considerable amino acid sequence similarity and inhibit protein synthesis by specifically cleaving a single phosphodiester bond in 23 S and 28 S rRNA of prokaryotic and eukaryotic ribosomes [17,18]. The fungal ribotoxins do not bind to any cell-surface receptors but manifest a potent cytotoxic activity if introduced inside the cell by artificial means [19]. Restrictocin has been successfully employed to make active immunotoxins [20–24]. Its small size, poor immunogenicity, absence of a cell-binding activity and thermostability make it a desirable candidate for constructing chimaeric toxins [24].

We have recently developed restrictocin-based chimaeric toxins by placing transforming growth factor α or the single-chain-antigen-combining region (scFv) of an anti-(human transferrin receptor) (anti-TFR) antibody separately at the N-terminus and

C-terminus of the toxin [25,26]. Although both N-terminal and C-terminal fusions had similar ribonucleolytic activity *in vitro*, cell-surface binding activity and intracellular routing, their cytotoxic activities were remarkably different [26]. Chimaeric toxins containing ligand at the C-terminus of the restrictocin were found to be more potent than those that had ligand at the N-terminus of the toxin [25,26]. It was therefore shown that the preferred site of direct ligand attachment on restrictocin was its C-terminus, which might become a limitation for employing restrictocin universally with any potential ligand for the construction of chimaeric toxins [25,26]. By using protease inhibitors it has been shown that, after internalization, restrictocin-based chimaeric toxins are cleaved proteolytically [26]. The difference between the cytotoxic activities of N-terminal and C-terminal fusion proteins was therefore proposed to be due to a difference in their intracellular proteolytic processing [26].

Here we describe the construction and characterization of second-generation chimaeric toxins comprising restrictocin and anti-TFR(scFv). Anti-TFR(scFv)–restrictocin and restrictocin–anti-TFR(scFv) have been further engineered to enhance their cell-killing activity. To facilitate folding and, in turn, the intracellular processing of restrictocin-containing chimaeric toxins, we have incorporated between the ligand and the toxin either a flexible peptide linker or a proteolytically cleavable spacer. The study demonstrates that with a proteolytically cleavable spacer, ligand can be placed at either end of restrictocin and that restrictocin-based chimaeric toxins containing a proteolytically cleavable spacer between the toxin and the ligand are much more potent.

EXPERIMENTAL

Construction of plasmids

All gene fusions were cloned in a phage T7 promoter-based *Escherichia coli* expression vector, pVex11. pVex11 is a pET-derived vector that was kindly provided by Professor V. K. Chaudhary (University of Delhi, Delhi, India). pRestrictocin–anti-TFR(scFv) (pRFv) and pAnti-TFR(scFv)–restrictocin (pFvR) were used as parent vectors for further manipulations [26]. The primers were designed to introduce the linker or the spacer by PCR. *E. coli* strain DH5α was used to propagate and manipulate plasmid DNA. The plasmids having the required gene fusions were identified by restriction analysis and protein expression. The presence of the linker and the spacer was confirmed by DNA sequencing with Sanger's method [27].

pRestrictocin–linker–anti-TFR(scFv) (pRLFv)

A linker, coding for the pentapeptide Gly-Gly-Gly-Gly-Ser, was incorporated between the restrictocin and scFv genes. The linker sequence was introduced at the 3′ end of the restrictocin gene by PCR with a previously described plasmid, pRest [28], as the template, which contains the restrictocin gene under T7 promoter as an *Nde*I–*Eco*RI fragment. The forward primer XUP (5′-ACTCACTATAGGGAGACCAC-3′) annealed upstream of the restrictocin-encoding gene in the promoter region of the vector. The reverse primer A2 (5′-GTCATCGTACT<u>CATATG</u>GGAT-CCCCCACCGCCATGAGAACACAG-3′) was designed to provide a DNA sequence encoding a Gly$_4$Ser linker at the 3′ end of restrictocin and an *Nde*I restriction site. The amplified fragment, restrictocin–linker, was digested with *Nde*I and ligated into plasmid pRFv, digested with the same enzyme, in place of restrictocin to generate pRestrictocin–linker–anti-TFR(scFv).

pAnti-TFR(scFv)–linker–restrictocin (FvLR)

The DNA encoding the pentapeptide linker was incorporated at the 5′ end of the restrictocin gene by PCR with pRest as the template. The forward primer A1 (5′-ATGTCTGTAG<u>CATATG</u>GGCGGGGGTGGATCCGCGACCTGGACATGC-3′) provided the linker sequence and an *Nde*I site; the reverse primer JSR3 (5′-TGTTAGCAGCC<u>GAATTC</u>AATGAGAACACAG-3′), containing an *Eco*RI site, annealed downstream of the restrictocin-encoding gene in the vector. Linker–restrictocin, obtained after PCR, was digested with *Nde*I and *Eco*RI and cloned into the vector pFvR, digested with the same enzymes, in place of the restrictocin fragment [26].
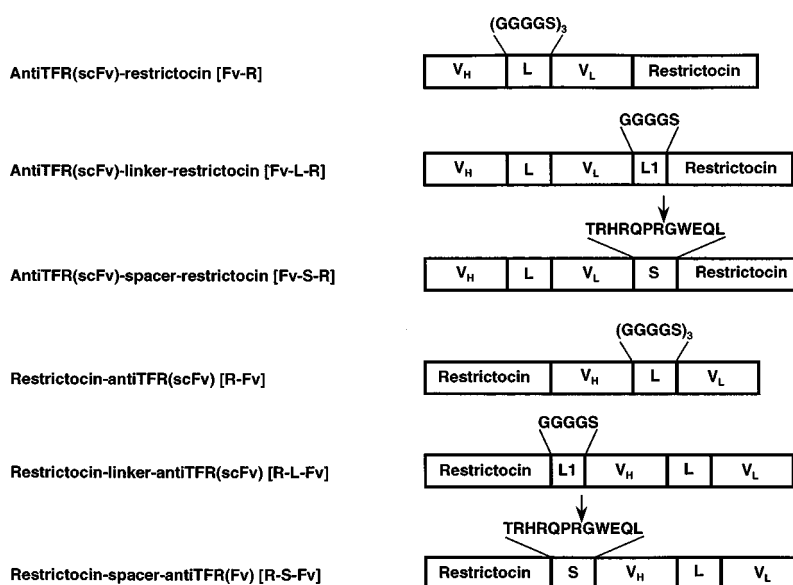
pRestrictocin–spacer–anti-TFR(scFv)

A spacer sequence encoding amino acid residues Thr-Arg-His-Arg-Gln-Pro-Arg-Gly-Trp-Glu-Gln-Leu was engineered between restrictocin and anti-TFR(scFv) to provide a protease recognition site. The 36 bp spacer was introduced at the 3′ end of restrictocin by two consecutive PCRs with pRest as the template in the first PCR, and the product of the first PCR as the template in the second PCR. In the first PCR, a sequence encoding the first eight amino acids of spacer was incorporated and the spacer was extended to its full length in the second PCR. Primer XUP (sequence mentioned above) was the forward primer for both the first and the second PCR and the reverse primers used were: A3 (5′-GCCTCGAGGCTGGCGATGACGGGTATGAGAAC-ACAG-3′) for the first PCR and A4 (5′-TGATTAGATACT<u>CATATG</u>CAGTTGTTCCCAGCCTCG AGGCTG-3′) for the second PCR. After the second PCR, the amplified fragment contained the gene encoding restrictocin with the 36 bp spacer at its 3′ end. The fragment was digested with *Nde*I and ligated into pRFv, digested with the same enzyme, in place of the restrictocin fragment.

pAnti-TFR(scFv)–spacer–restrictocin (FvSR)

The spacer coding sequence was incoporated at the 5′ end of the restrictocin gene by two consecutive PCRs with JSR3 as the reverse primer and the following forward primers: DR3 (5′-CAGCCGCGCGGCTGGGAACAACTGGCGACCTGG-ACA-3′) for the first PCR and DR4 (5′-ATCTTACGC-<u>CATATG</u>ACCCGTCATCGCCAGCCGCGCGGC-3′) for the second PCR. pRest was the template in the first PCR; the product of the first PCR was used as the template in the second. The forward and reverse primers provided respectively an *Nde*I and an *Eco*RI site on the 5′ and 3′ ends of the spacer-containing restrictocin fragment. The amplified fragment was digested with *Nde*I and *Eco*RI and ligated into pFvR, digested with the same enzymes, in the place of the restrictocin fragment.

Expression and purification of chimaeric toxins

A competent BL21(λDE3) strain of *E. coli* cells was transformed separately with different constructs and grown in superbroth at 37 °C containing 100 μg/ml ampicillin. The cultures were induced, at a D_{600} of 2.0, with 1 mM isopropyl β-D-thiogalacto-pyranoside for 2 h. The fusion proteins were overexpressed and were found to localize in inclusion bodies. The proteins from inclusion bodies were purified as described [29]. The inclusion bodies were denatured with 6 M guanidinium chloride, reduced with dithioerythritol and renatured in a buffer containing arginine and GSSG. The renatured protein, after dialysis in 20 mM Mes, pH 5.5, was loaded on an SP-Sepharose column (Pharmacia)

Figure 1   Schematic representation of restrictocin-based chimaeric toxins

In RLFv and FvLR a flexible peptide linker (L1), and in RSFv and FvSR a proteolytically cleavable spacer sequence (S), is incorporated between restrictocin and anti-TFR(scFv) in the chimaeric toxins. L, linker between the variable heavy chain and the variable light chain of anti-TFR antibody. Amino acids (single-letter codes) in bold capitals represent the connecting peptide sequences. The proteolytic cleavage site in the spacer is shown by an arrow.

and eluted with a 0–1 M NaCl gradient on an FPLC system (Pharmacia). Proteins were further purified by gel-exclusion chromatography on a TSK 3000SW column and analysed by SDS/PAGE [12% (w/v) gel] under reducing conditions. The concentration of purified proteins was estimated by Bradford's method [30] with Coomassie plus reagent (Pierce). SDS/PAGE was performed by the method of Laemmli [31].

Characterization of proteins by CD spectroscopy

CD spectra of proteins dissolved in 50 mM sodium phosphate buffer, pH 7.4, were recorded in the far-UV range at 25 °C with a Jasco J710 spectropolarimeter.

Cell-free assay of inhibition of protein synthesis

Ribonucleolytic activity of restrictocin, in chimaeric toxins, was assessed in a translation assay *in vitro* containing rabbit reticulocyte lysate. Rabbit reticulocyte lysate was prepared, and assay done, as described [32]. Various concentrations of chimaeric toxins and restrictocin were incubated with rabbit reticulocyte lysate for 1 h at 30 °C; the reaction was terminated by the addition of 1 M NaOH containing H_2O_2. The translated product was precipitated with 20% (w/v) trichloroacetic acid and the radiolabelled protein was harvested on glass fibre filters and counted in a liquid-scintillation counter.

Assay of cytotoxicity and specificity of chimaeric toxins

Protein synthesis was measured in a variety of cell lines in the absence or presence of the toxins. All cell lines were maintained in RPMI medium containing 10% (v/v) fetal calf serum. Adherent cells were plated in 96-well flat-bottomed tissue culture plates at 5×10^3 cells per well and allowed to adhere overnight at 37 °C. Next day, the medium was removed and replaced with 200 μl of leucine-free RPMI medium containing 10% serum.

Suspension cells were seeded at 5×10^3 cells per well in leucine-free RPMI medium containing 2% (v/v) fetal calf serum and used immediately. Serial dilutions of proteins, made in PBS containing 0.2% (v/v) human serum albumin, were added to the cells, incubated for the indicated durations and then pulsed with [³H]leucine (1 μCi per well) for 3 h. Cells were harvested on filter mats and the incorporation of [³H]leucine into cellular protein was assayed with an LKB Beta-Plate counter. To check the specificity of chimaeric toxins for TFR, 10 μg of anti-TFR antibody (HB21) was added to each well before the addition of fusion protein in the competition experiments.

Binding studies

Competition binding analysis was performed to compare the affinity of the chimaeric toxins with the native antibody. Anti-TFR antibody (HB21) was iodinated by the Iodogen method [33]. Adherent A549 and A431 cells were seeded at 4×10^5 cells per well and used 16 h later for the assay. HUT102 cells were also plated at the same density and used immediately. After two washes with binding buffer (0.1% BSA in Dulbecco's modified Eagle's medium), various dilutions of toxin, along with 3 ng of labelled antibody in binding buffer, were added to the cells. The cells were incubated at 25 °C for 2 h with mild shaking, washed three times with binding buffer and lysed in 10 mM Tris/HCl (pH 7.4)/1 mM EDTA/0.5% SDS. The radioactivity associated with the cells was counted in a γ-counter (LKB).

RESULTS

Construction of chimaeric toxins

We have previously developed two chimaeric toxins, anti-TFR(scFv)–restrictocin and restrictocin–anti-TFR(scFv), in which the Fv portion of a monoclonal antibody against human TFR was genetically fused respectively at the N-terminus and the
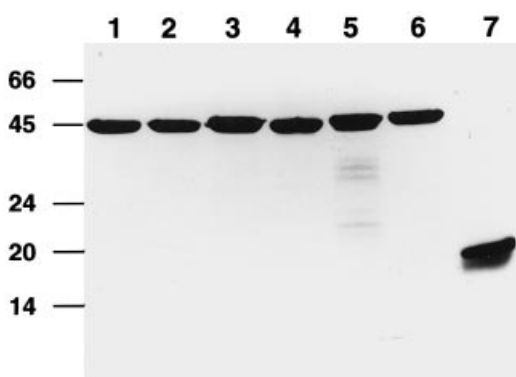
Figure 2   SDS/PAGE of purified restrictocin-based chimaeric toxins

The purified proteins were analysed by SDS/PAGE [12.5% (w/v) gel] stained with Coomassie Blue. The positions of molecular mass markers are indicated (in kDa) at the left. Lane 1, FvR; lane 2, FvLR; lane 3, FvSR; lane 4, RFv; lane 5, RLFv; lane 6, RSFv; lane 7, restrictocin.

C-terminus of ribonucleolytic toxin restrictocin [26]. Restrictocin–anti-TFR(scFv) was more active than anti-TFR(scFv)–restrictocin on all the target cell lines [26]. It was therefore concluded that for restrictocin-based chimaeric toxins to be optimally active, ligand should be placed at the C-terminus of the toxin. To improve the activity of restrictocin-based chimaeric toxins, anti-TFR(scFv)–restrictocin and restrictocin–anti-TFR(scFv) were further engineered by incorporating between ligand and toxin (1) a flexible peptide linker Gly-Gly-Gly-Gly-Ser or (2) a proteolytically cleavable spacer Thr-Arg-His-Arg-Gln-Pro-Arg-Gly-Trp-Glu-Gln-Leu. The proteolytically cleavable spacer used in this study was derived from domain II of PE; PE is cleaved intracellularly by a protease within this sequence between Arg and Gly, to generate a 37 kDa translocation-competent fragment [9,34]. On the basis of biochemical studies a serine protease, furin, has been proposed to be responsible for this cleavage [11,35–38]. PE is also cleaved *in vitro* by trypsin at the same location to produce the 37 kDa fragment

[34,37]. A schematic representation of gene fusions encoding FvLR, FvSR, restrictocin–linker–anti-TFR(scFv) (RLFv) and restrictocin–spacer–anti-TFR(scFv) (RSFv) is shown in Figure 1.

The gene fusions were cloned in a T7 promoter-based *E. coli* expression vector. After expression in *E. coli*, the proteins accumulated in the form of inclusion bodies within the cell. The recombinant proteins were isolated from the inclusion bodies by denaturation in guanidinium hydrochloride; after renaturation they were purified to homogeneity by successive cation-exchange and gel-filtration chromatography (Figure 2).

To investigate the effect of introducing a flexible peptide linker or a cleavable spacer between the ligand and restrictocin on their conformation, the chimaeric toxins were analysed by CD spectroscopy. Restrictocin–anti-TFR(scFv) (RFv), previously shown to have a potent cytotoxic activity, seemed to have a compactly folded $\alpha + \beta$ structure (Figure 3A), whereas RLFv and RSFv, although similar to each other, were significantly different in conformation from RFv (Figure 3A). Among the chimaeric toxins in which the ligand was placed on the N-terminus of restrictocin, FvLR seemed to be compactly folded similarly to RFv; however, anti-TFR(scFv)–restrictocin (FvR) and FvSR were not optimally folded (Figure 3B).

Effect of chimaeric toxins on protein synthesis *in vitro*

Previously we found that fusing a ligand either at the N-terminus or at the C-terminus of restrictocin drastically affected the ribonucleolytic activity of restrictocin: the chimaeric proteins were approx. 1/30 as active as the native toxin [26]. To investigate the effect of introducing a spacer or a linker between the ligand and the toxin on the catalytic activity, the ribonucleolytic activity of chimaeric toxins was tested in a translation assay *in vitro* containing rabbit reticulocyte lysate. All constructs inhibited protein synthesis in a dose-dependent manner, with doses giving half-maximal inhibition (ID_{50}) ranging between 1 and 2 nM, in comparison with 0.07 nM for restrictocin (Table 1). There was no significant improvement in the ribonucleolytic activity *in vitro* of restrictocin containing chimaeric toxins as a result of the incorporation of a flexible linker or a cleavable spacer between the ligand and the toxin (Table 1).
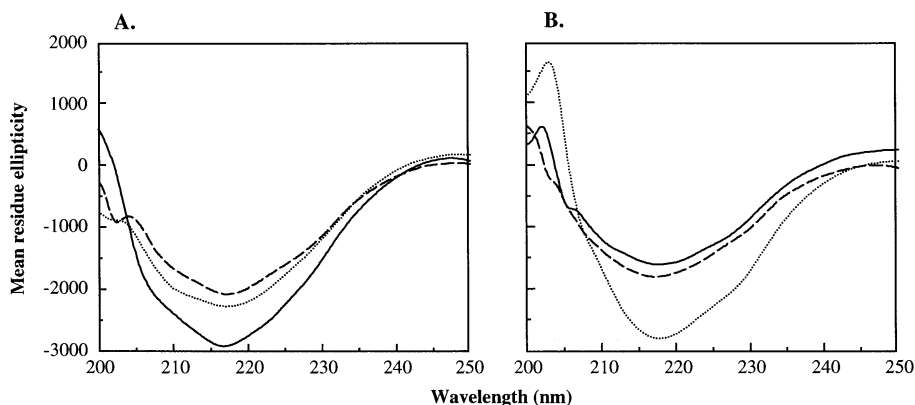
Figure 3   CD-spectral analysis of restrictocin-based chimaeric toxins

A cell with a 1 cm path was used to record the spectra of proteins. Spectra were acquired at a scan speed of 50 nm/min with a sensitivity of 0.050° and a response time of 1 s. The samples were purged with nitrogen and spectra were averaged over 10 accumulations. Mean residue ellipticity is expressed in units of m-degrees·cm^2·$dmol^{-1}$. (**A**) Solid line, RFv; dotted line, RLFv; broken line, RSFv. (**B**) Solid line, FvR; dotted line, FvLR; broken line, FvSR.

Table 1   Effect of restrictocin-based chimaeric toxins on translation *in vitro*

Rabbit reticulocyte lysate was treated with various concentrations of different proteins at 30 °C for 1 h. Protein synthesis was determined by measuring the incorporation of [^{3}H]leucine into newly synthesized proteins.

Chimaeric toxin	ID$_{50}$ (nM)
FvR	1.50
FvLR	1.01
FvSR	0.88
RFv	2.05
RLFv	2.25
RSFv	0.91
Restrictocin	0.07

Table 2   Cytotoxicity of restrictocin-based chimaeric toxins on various cancer cell lines

Human cancer cell lines HUT102 (T-cell leukaemia), K562 (erythroleukaemia), A549 (lung carcinoma), COLO205 (colon adenocarcinoma), MCF7 (breast adenocarcinoma), A431 (epidermoid carcinoma) and HeLa (cervical carcinoma), and a mouse fibroblast cell line, L929, were used.

| Cell line | ID$_{50}$ (nM) | | | | | |
	FvR	FvLR	FvSR	RFv	RLFv	RSFv
HUT102	0.17	0.02	0.006	0.06	0.07	0.005
K562	1.14	0.14	0.12	0.14	0.54	0.08
A549	0.80	0.23	0.13	0.14	0.29	0.10
COLO205	1.45	0.23	0.13	0.27	0.45	0.14
MCF7	2.27	0.47	0.22	0.36	0.67	0.16
A431	5.00	0.38	0.16	0.61	0.83	0.40
HeLa	8.20	0.82	0.89	0.98	2.20	1.10
L929	> 114	> 112	> 111	> 114	> 112	> 111
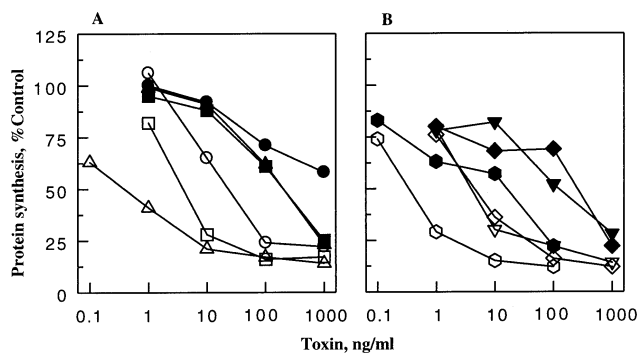
Figure 4   Toxicity of chimaeric toxins towards HUT102 cell line

The cytotoxic activity of chimaeric toxins is plotted as the percentage inhibition of protein synthesis relative to the untreated control after 48 h. Cells were incubated with various concentrations of chimaeric toxins as follows: (**A**) N-terminal constructs FvR (○,●), FvLR (□,■) or FvSR (△,▲); (**B**) C-terminal constructs RFv (▽,▼), RLFv (◇,◆) or RSFv (⬡, ⬣). The open and closed symbols represent the chimaeric toxin tested in the absence and the presence respectively of excess of anti-TFR antibody.

Cytotoxicity and specificity of chimaeric toxins

The cytotoxic activity of chimaeric toxins was tested on a variety of human cancer cell lines overexpressing TFR. The fusion proteins inhibited protein synthesis in the target cell lines in a dose-dependent manner (Figure 4 and Table 2). As shown in Figure 4 for HUT102 cells, the addition of an excess of anti-TFR antibody blocked the manifestation of toxicity by these fusion proteins. The incomplete neutralization observed at the highest chimaeric toxin concentrations seemed to be due to a lower antibody-to-toxin ratio. Similar results were obtained on all target cell lines studied, demonstrating that all chimaeric toxins were specifically recognizing the TFR. All fusion proteins were non-toxic to a mouse fibroblast cell line, L929, producing no inhibition of protein synthesis even at 112 nM, indicating the species-specific binding of the proteins to TFR. Restrictocin alone, up to 100 nM, did not inhibit protein synthesis in any of the cell lines studied (results not shown). All chimaeric toxins exhibited maximum activity on HUT102, a T-cell leukaemia cell line (Table 2). FvSR and RSFv were 28-fold and 12-fold more cytotoxic than FvR and RFv respectively (Table 2). FvLR, in comparison with that of FvR, showed a 9-fold higher cytotoxicity

towards the same cell line. The incorporation of the Gly-Gly-Gly-Gly-Ser linker between Fv and restrictocin resulted in a 3–13-fold increase in the activity of Fv-restrictocin on all cell lines tested (Table 2). In contrast, RLFv was found to have an activity that was either marginally decreased or similar to that of RFv on all target cell lines (Table 2). The incorporation of the cleavable spacer Thr-Arg-His-Arg-Gln-Pro-Arg-Gly-Trp-Glu-Gln-Leu improved the activity of FvR remarkably and that of RFv marginally on all cell lines (Table 2). FvSR and RSFv had almost similar cytotoxic activities towards all target cells (Table 2).

Binding of chimaeric toxins to TFR

To check whether the differences in cytotoxicity of various proteins were due to a difference in the binding affinities of these constructs for TFR, the binding activities of these chimaeric toxins were compared with that of native antibody towards HUT102, A431 and A549 cells. All the proteins were found to be equally potent in displacing the native anti-TFR monoclonal antibody from the three cell lines studied (Figure 5). Restrictocin, used as a non-specific control, showed no competition with native antibody even at the 100-fold higher concentration (results not shown).

Susceptibility of chimaeric toxins to trypsin-mediated cleavage

From our previous study it was evident that, to manifest their cytotoxic activity, restrictocin-based chimaeric toxins needed to be processed proteolytically [26]. In the present study it was found that the incorporation of a proteolytically cleavable spacer in these chimaeric toxins improved their cytotoxicity significantly. To investigate the susceptibility of various chimaeric toxins to proteolytic cleavage, and to locate the cleavage site, the fusion proteins were treated with trypsin at pH 7.4 for various durations. Two major fragments, of 27 and 16 kDa, were obtained by a limited trypsin digestion of spacer-containing chimaeric toxins (Figure 6A). The 16 kDa fragment, corresponding to restrictocin, was also obtained from FvR and RFv, but in a very small quantity, indicating a relatively inefficient processing of these proteins (Figure 6A). The digestion of the spacer-containing proteins was complete within 15 min, whereas all other
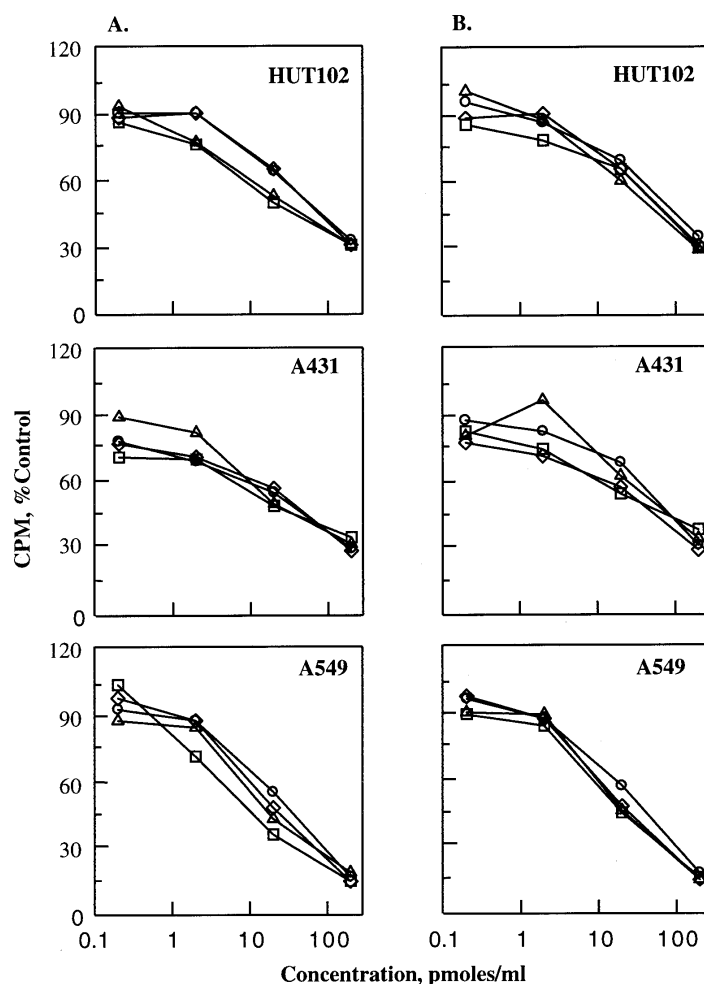
Figure 5   Binding of chimaeric toxins to target cell lines

Iodinated anti-TFR antibody mixed with various concentrations of the unlabelled antibody or various chimaeric toxins was incubated with indicated cell line. Binding analyses was performed with the following: (**A**) N-terminal constructs FvR (△), FvLR (○) or FvSR (□) or unlabelled antibody (◇); (**B**) C-terminal constructs RFv (△), RLFv (○) or RSFv (□) or unlabelled antibody (◇).

fusion proteins were comparatively resistant to the protease: even after a prolonged incubation only a very faint 16 kDa band was detected (results not shown). However, no non-specific degradation was visible even after prolonged treatment with trypsin. A similar pattern was observed when the proteins were digested at pH 5.25 (results not shown).

The fragments produced were characterized by Western blotting by probing with an anti-restrictocin antibody. The 16 kDa band released from RSFv and FvSR corresponded to restrictocin (Figure 6B). After digestion, RLFv produced a band that reacted with anti-restrictocin antibody and had a higher molecular mass than that of restrictocin and the 16 kDa band released from RSFv and FvSR (Figures 6A and 6B). The site of cleavage in spacer-containing chimaeric toxins was ascertained by N-terminal sequence analysis of the two fragments obtained; the cleavage was found to be occurring precisely at the predicted cleavage site producing the 27 kDa scFv fragment and 16 kDa restrictocin fragment (results not shown).

Kinetics of intoxication by restrictocin-containing chimaeric toxins

To investigate the kinetics of intoxication by different restrictocin-containing chimaeric toxins, their cytotoxic activity was assayed

on HUT102, K562 and A431 cells at various time points (Table 3). The cell lines that were more sensitive to the spacer-containing or linker-containing chimaeric toxins were used as model cell lines for this study. As reported previously, FvR and RFv showed potent cytotoxicity only after 36–48 h, whereas RSFv and FvSR, chimaeric toxins containing the cleavable spacer, manifested potent cytotoxic activity starting at 24 h (Table 3). This shift in kinetics was observed for all the cell lines studied (Table 3), indicating that the protease responsible for the cleavage of these chimaeric toxins was present ubiquitously. In addition, with the protease cleavage site present in the chimaeric toxins, they were processed efficiently to release a translocation-competent active fragment of restrictocin intracellularly. Of the linker-containing proteins, FvLR also showed the peak activity earlier; however, the kinetics of RLFv was similar to that of RFv (Table 3).

DISCUSSION

Restrictocin, a member of the fungal ribotoxin family, is a promising candidate for the construction of immunotoxins and chimaeric toxins [20–22,24–26]. Ribotoxins' inability to enter the normal cells, their stability and their low immunogenicity are
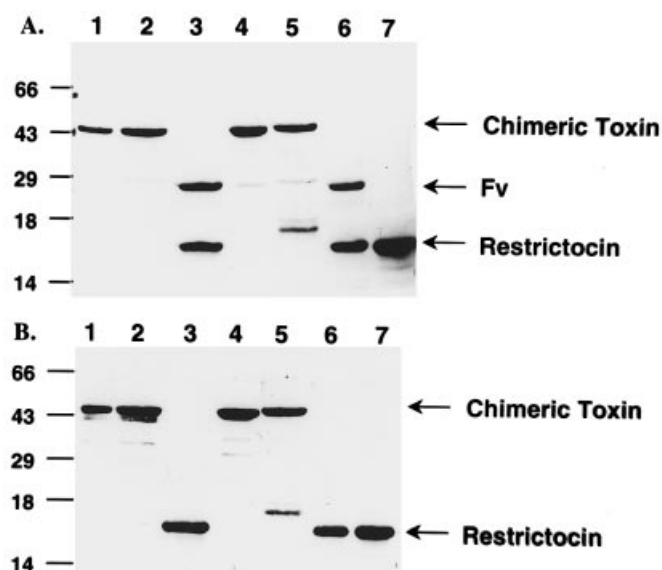
Figure 6 Sensitivity of restrictocin-based chimaeric toxins to trypsin-mediated cleavage

Restrictocin and restrictocin-based chimaeric toxins were digested in 25 mM Hepes, pH 7.4, containing 1 mM CaCl$_2$ and 0.5 mM EDTA. Trypsin (500 ng) was added to 1 μg of protein and the samples were incubated at 37 °C for 30 min. The reaction was terminated by the addition of SDS/PAGE sample buffer. The samples were analysed by SDS/PAGE on 12% (w/v) reducing gels and detected by staining with Coomassie Blue. (**A**) SDS gel stained with Coomassie Blue. (**B**) Western blot probed with a polyclonal anti-restrictocin antibody. Lanes 1, FvR; lanes 2, FvLR; lanes 3, FvSR; lanes 4, RFv; lanes 5, RLFv; lanes 6, RSFv; lane 7, restrictocin. The positions of molecular mass markers are indicated (in kDa) at the left. The ligand, anti-TFR(scFv) and restrictocin fragments produced as a result of digestion of the restrictocin-based intact chimaeric toxins are indicated by arrows at the right.

some of the desirable characteristics for their use in the construction of chimaeric toxins. Gasset et al. [39,40] have shown that α-sarcin interacts with negatively charged phospholipid vesicles, which could be a mechanism for its intracellular translocation. The cytotoxic activity of restrictocin containing chimaeric toxins and immunotoxins supports the contention that restrictocin is translocation-competent [20–26]. The crystal structure of restrictocin revealed that the surface of the molecule is dome-shaped and that the active site is located on the planar side [41]. The N-terminus and C-terminus of the molecule are linked by a disulphide bond (Cys^5–Cys^{147}) [41]. The structure therefore

does not put any apparent constraint on putting a ligand on either of the termini of restrictocin for developing a chimaeric toxin. However, it has been shown previously that the chimaeric toxins with a ligand at the C-terminus were invariably more active on all the cell lines studied than their N-terminal counterparts [25,26]. These studies implied that the differential cytotoxicity was due to (1) a difference in the intracellular processing and/or (2) a difference in the translocation of the enzymically active moiety in the cytosol. The difference in activity could also be attributed to simple differences in folding of the two constructs. The present study was aimed at designing restrictocin-based chimaeric toxins with improved cytotoxic activity and to make restrictocin a universal toxin such that a ligand could be placed at either of the two termini without compromising the activity. A linear flexible linker, Gly-Gly-Gly-Gly-Ser, was incorporated between the ligand [anti-TFR(scFv)] and the toxin (restrictocin) to promote the independent folding of the two proteins in the chimaera. The introduction of the linker improved the activity of only the chimaera in which the ligand was attached at the N-terminus of restrictocin and made it as active as RFv, the chimaera with the ligand at the C-terminus of restrictocin. Thus the ligand could also be attached at the N-terminus of restrictocin with an intermediary linker sequence. However, RLFv had a decreased activity in comparison with FvR, indicating that the linker was detrimental to the activity of RFv and that the linker could not be used universally at either end to construct chimaeric toxins with restrictocin. The conformations of RFv and FvLR, which had similar cytotoxic activities, were also very similar, indicating that an improvement in the activity with the incorporation of the linker was due to an improvement in the folding of the molecule.

It was demonstrated previously that a non-cleavable immunotoxin, generated by chemically coupling a monoclonal antibody against human TFR to restrictocin through a stable linkage, was inactive on most of the cell lines, whereas the cells were sensitive towards a cleavable conjugate constructed from the same antibody and restrictocin [24]. In addition, in the presence of the protease inhibitor tosyl-lysylchloromethane ('TLCK'), the cytotoxicities of FvR and RFv were significantly diminished, indicating the involvement of a protease in the cytotoxicity of these chimaeric toxins [26]. Recombinant chimaeric toxins containing PE and DT are processed proteolytically to produce translocation-competent active fragments to manifest their cytotoxicity [9–11]. A cellular protease, furin, is responsible for the proteolytic processing of these toxins [11]. The furin recognition sequence in PE is present within the disulphide loop between Cys^{267} and Cys^{287} in the translocation domain [9,34]. In the

Table 3 Kinetics of cytotoxicity of restrictocin-based chimaeric toxins

HUT102, K562 and A431 cells, at a density of 5×10^4 cells/ml, were incubated with different concentrations of chimaeric toxins for indicated durations; protein synthesis was assayed by measuring the incorporation of [^{3}H]leucine into newly synthesized protein.

Chimaeric toxin	Duration (h)...	ID$_{50}$ (ng/ml)								
		HUT102			K562			A431		
		24	36	48	24	36	48	24	36	48
FvR		150	7	16	> 1000	600	70	> 1000	550	500
FvLR		20	1.5	2.5	100	30	4.5	220	60	16
FvSR		5	0.5	0.8	57	22	4.2	200	25	10
RFv		37	2	3.5	350	35	13	600	100	60
RLFv		55	25	5	250	42	18	800	140	140
RSFv		1	0.3	0.3	35	13	4.5	250	60	40

present study we incorporated the peptide sequence Thr-Arg-His-Arg-Gln-Pro-Arg-Gly-Trp-Glu-Gln-Leu from PE, containing the furin recognition site, between the ligand and the toxin in restrictocin-containing chimaeric toxins to facilitate the intracellular processing of these proteins. The inclusion of the proteolytically cleavable spacer resulted in a marked increase in the activity of both types of chimaeric toxin, indicating that processing is important in the manifestation of activity by these chimaeric toxins. RSFv and FvSR had similar cytotoxicities towards all the cell lines studied and they were equally active as, or more active than, RFv. *In vitro*, both RSFv and FvSR were processed in a similar manner by trypsin. It is seen that the cytotoxicity is manifested by the chimaeric toxins from which a full-length (16 kDa) restrictocin fragment is released *in vitro* by digestion with trypsin. RLFv produced a fragment that was larger than the restrictocin fragment, and correspondingly the cytotoxicity of this chimaera was much lower. Without a spacer or a linker, although the fusion proteins are processed to release the restrictocin fragment, the processing seems to be much less efficient. A shift in the kinetis of intoxication by the spacer-containing chimaeric toxins also shows that efficient intracellular processing is required for restrictocin-based immunotoxins to be optimally active. The study demonstrates that for restrictocin-containing chimaeric toxins to be active they need to be folded such that a full-length restrictocin fragment is released from them by intracellular proteolytic processing. The proteolytically cleavable spacer-containing chimaeric molecules are processed efficiently and precisely within the spacer, although from the CD spectral analysis they seem not to be optimally folded. The furin recognition sequence might be exposed on the surface in these chimaeric toxins and therefore accessible to the protease. Previously, chimaeric toxins have been produced containing the ricin A chain and Protein A linked via a trypsin-sensitive spacer sequence from DT, to produce disulphide-linked ricin A chain and Protein A by proteolytic activation [12]. Although this chimaera is active, it needs to be nicked proteolytically *in vitro* before its addition to the target cells [12]. The proteolytically cleavable spacer sequence used in this study is efficiently processed by all the cell lines studied without any prior activation *in vitro*.

In conclusion, we have designed and developed the next generation of restrictocin-containing immunotoxins with improved biological activity; it should now be possible to use restrictocin for the construction of immunotoxin with any potential targeting ligand.

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