Self-potentiation of Ligand-Toxin Conjugates Containing Ricin A Chain Fused with Viral Structures*

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A chimeric protein was obtained by fusing together the ricin toxin A chain (RTA) gene and a DNA fragment encoding the N terminus of protein G of the vesicular stomatitis virus. Chimeric RTA (cRTA) retained full enzymic activity in a cell-free assay, but was 10-fold less toxic against human leukemic cells than either native RTA (nRTA) or unmodified recombinant RTA (rRTA). However, conjugates made with cRTA and human transferrin (Tfn) showed 10-20-fold greater cell killing efficacy than Tfn-nRTA or Tfn-rRTA conjugates despite equivalent binding of the three conjugates to target tumor cells. As a consequence, by fusion of the KFT25 peptide to the RTA sequence, the specificity factor (i.e. the ratio between nonspecific and specific cytotoxicity) of Tfn-cRTA was increased 90–240 times with respect to those of Tfn-nRTA and Tfn-rRTA. cRTA interacted with phospholipid vesicles with 15-fold faster kinetics than nRTA at acidic pH. Taken together, our results suggest that the ability of vesicular stomatitis virus protein G to interact with cell membranes can be transferred to RTA to facilitate its translocation to the cell cytosol. Our strategy may serve as a general approach for potentiating the cytotoxic efficacy of antitumor immunotoxins.

Cell-surface structures mediating the efficient internalization of cell-bound molecules are frequently selected as targets of monomodal antibody/ligand-toxin conjugates (immunotoxins (IT)) (1). Rapid internalization, however, is not always synonymous with fast intoxication rates of the target cells as a result of cell mechanisms leading to inactivation of the internalized IT molecules (i.e. recycling, degradation, slow routing to subcellular compartments competent for toxin translocation) (1). The ricin toxin A chain (RTA) is a potent ribosome-inactivating enzyme used in the synthesis of highly selective IT. However, RTA-based IT exert their effect at relatively high concentrations due to poor translocation of RTA to the cell cytosol from the endocytic compartments where the IT are internalized (1).

Viruses utilize specialized envelope structures that allow them to enter the cytosol of the infected cells. We reasoned that it might be possible to modify a cytotoxic enzyme (i.e. RTA) by fusing it to a protein structure derived from viral envelopes, thus conferring to the cytotoxic enzyme the cytosol targeting properties of the virus. A peptide representing the primary sequence of the 25 N-terminal amino acids of protein G of the vesicular stomatitis virus envelope (KFT25) was found to have pH-dependent membrane destabilizing properties (2, 3). In particular, at low pH, KFT25 was shown to be hemolytic, to mediate hemaggutination, to be cytotoxic for mammalian cells, and to effect gross changes in cell permeability (2, 3). Such a virus-derived structure might be endowed with the ability to facilitate the translocation of heterologous proteins across cell membranes when they are routed to acidic intracellular compartments.

The transferrin receptor is a cell-surface structure known to deliver internalized protein-protein conjugates to acidic compartments (i.e. endosomes) (4, 5). The physiology of the transferrin receptor and of its ligand has been well studied, and Tfn-toxin conjugates have found applications in the laboratory as well as in the clinic as antitumor reagents (6, 42). Internalized Tfn and Tfn-toxin conjugates are directed to acidic prelysosomal compartments within the cell (4, 5). Tfn was therefore chosen as an appropriate vehicle molecule to investigate whether RTA cell entry would be improved by fusion with KFT25.

In this preliminary report, we show that a KFT25-containing RTA (chimeric RTA (cRTA)) exhibits a greater cytotoxic activity when delivered to tumor cells by Tfn than analogous conjugates containing either native RTA (nRTA) or unmodified recombinant RTA (rRTA). These results open up the possibility of taking advantage of specialized viral structures to increase the cytosolic localization of toxins or other biologically active proteins within target cells.

EXPERIMENTAL PROCEDURES

Cloning and Expression of Recombinant Toxins—The vector pRICA, designed for expression of active RTA in the cytoplasm of Escherichia coli, has been described elsewhere (7). The RTA coding region with an additional 26 base pairs at the 5'-end is contained in an 873-base pair BamHI fragment. Within the 26-base pair extrasequence and immediately downstream from the BamHI site, an XbaI restriction site is present. An XbaI-BamHI fragment containing the RTA sequence was isolated and ligated with a BamHI-XbaI oligonucleotide coding for the 25 N-terminal amino acids of vesicular stomatitis virus protein G into
mediated transformation. Cultures were grown at 37°C in 1 liter of M9 5 mM phosphate buffer (pH 6.5) and passed through an ion-exchange column (2.5 × 12 cm) of CM-Sepharose equilibrated in dialysis buffer. Bound proteins were eluted with a 0–500 mM NaCl gradient, and fractions were then analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The purity of RTA- and cRTA-containing fractions was >90% as evaluated by scanning densitometry with a GS-300 gel-scanning apparatus (Hoefer Scientific Instruments). The biological activity of purified rRTA and cRTA was determined by their ability to inhibit [35S]Met incorporation into proteins in a rabbit reticulocyte lysate (Boehringer Mannheim) and was compared with that of nRTA. nRTA was kindly provided by Dr. P. Casellas (Sanofi Recherche, Montpellier, France).

Preparation of Lipid Vesicles and Light Scattering Determinations—Small unilamellar vesicles (SUV) were prepared by sonication as described previously using egg phosphatidylcholine (PC) (Avanti Polar Lipids) and phosphatidylserine (PS) (Lipid Products) in a 1:1 molar ratio (9). Briefly, the dried lipid mixture was dissolved in chloroform, and solvent was evaporated under reduced pressure. Lipids at a concentration of 6 mg/ml were suspended in phosphate-buffered saline containing 5 mM isopropyl-d-thiogalactopyranoside (8 M in EtOH), experiments were carried out in separate dose-response assays; in each assay, a constant concentration of Tfn was present. Monensin was previously shown not to alter the binding of IT to target receptors (13). Monensin was used in binding studies because it greatly potentiates the cytotoxicity of Tfn-RTA IT, thus allowing us to test the inhibiting effect of a higher range of Tfn concentrations. From each set of triplicate 96-well plates containing the different concentrations of IT in enzyme units) required to give the same response in the presence (T) or absence (T) of the inhibitor (Tfn). Dose ratios (T/T) were calculated at a response level of 0.5 and plotted according to the method of Schild (14) against the log of the concentration of the inhibitor (T).

RESULTS AND DISCUSSION

Three forms of RTA were used in our experiments: nRTA (i.e. RTA prepared from native ricin that had been purified from the seeds of Ricinus communis), unmodified cRTA, and cRTA (recombinant RTA fused to KFT25). A cRTA cloning strategy was developed to preserve in the expressed cRTA molecule the N-terminal orientation possessed by the KFT25 peptide in vesicular stomatitis virus protein G (Fig. 1A). The insertion of the KFT25 DNA sequence at the 5’-end of the RTA coding region did not affect the expression level or the post-translational localization of cRTA with respect to RTA. With both pRI and pRAK25 expression vectors, the amount of toxin purified from bacterial cultures ranged from 1.5 to 2 mg/l. No cRTA was found into the periplasmic fraction, in the culture medium, or stored into inclusion bodies (data not shown). Fig. 1B shows the SDS-PAGE migration and Western blot identification of nRTA and cRTA following expression in E. coli and purification by ion-exchange chromatography. Control nRTA typically migrated in two distinct bands because of the

overnight at 4°C with 0.14 M dithiothreitol in order to break the S-S bond introduced between Tf and the toxins. This procedure was required because conjugated RTA is enzymically inactive (11). The full reduction of the S-S bond was monitored by SDS-PAGE analysis. The samples were then serially diluted and added to a rabbit reticulocyte lysate. Incorporation of [35S]Met into proteins was measured. Dithiothreitol did not interfere with the incorporation of the radiolabeled amino acid.

Cytotoxicity Assays—The effects of IT or of unconjugated toxin treatment on tumor cells were compared in protein synthesis inhibition assays. Protein synthesis was assayed by dispensing 10^3 j urkat cells in leucine-free, fetal bovine serum-free RPMI 1640 medium in 96-well flat-bottomed microtiter plates. Ten-fold dilutions of IT or of toxins were then added (final volume of 100 μl) in triplicates. After 6 h of incubation at 37°C, fetal bovine serum was added to each well to a final concentration of 2.5%. Microcultures were incubated for a further 16 h. After this time, the cells were pulsed for 2 h with 1 μCi of [3H]Leu (314.3 mCi mmol/μl; Dupont NEN). At the end of the assays, the cells were harvested onto glass-fiber filters, washed with water, and dried. Radioactivity incorporated by the cells was then measured in a β-spectrometer. When the cell killing activity of the IT was assayed in the presence of monensin (5 × 10^{-5} M in EOEH), experiments were carried out under the same general conditions described above with the difference that the assays were interrupted after 6 h.

Comparing Cytotoxicity of Free Toxins and of Their Tfn Conjugates on the Cytotoxic Activity of nRTA, cRTA, and rRTA and their Tfn conjugates, the concentration of the cytotoxic agents inhibiting 50% of the protein synthesis of target tumor cells (IC_{50}) was considered. However, rRTA, cRTA, and rRTA as well as Tfn-nRTA, Tfn-cRTA, and Tfn-rRTA displayed a similar but not identical enzymic activity in cell-free assays of protein synthesis inactivation (see “Results and Discussion”), and therefore, the molar concentrations of the different compounds could not be taken for direct comparison of their cytotoxic effect in cell killing experiments. Thus, we normalized the concentrations of nRTA, cRTA, and rRTA and of their Tfn conjugates by setting 1 enzyme unit (EU) as the IT or toxin molar concentration inhibiting 50% (IC_{50}) of the incorporation of [35S]Met in a rabbit reticulocyte lysate (9). In all the cytotoxicity assays, the IT or toxin concentrations are expressed in enzyme units.

Binding of Tfn-Toxin Conjugates to Tumor Cells—Binding of Tfn-nRTA, Tfn-cRTA, and Tfn-rRTA to urkat cells was evaluated following the method described by Ittelson and Gill (12). Briefly, dose-response assays with the three IT were carried out in the presence of monensin. The cytotoxicity of the IT was inhibited by various concentrations of unconjugated Tf (ranging from 0 to 3.6 × 10^{-8} M) in separate dose-response assays; in each assay, a constant concentration of Tfn was present. Monensin was previously shown not to alter the binding of IT to target receptors (13). Monensin was used in binding studies because it greatly potentiates the cytotoxicity of Tfn-RTA IT, thus allowing us to test the inhibiting effect of a higher range of Tfn concentrations. From each set of triplicate 96-well plates containing the different concentrations of IT in enzyme units) required to give the same response in the presence (T) or absence (T) of the inhibitor (Tfn). Dose ratios (T/T) were calculated at a response level of 0.5 and plotted according to the method of Schild (14) against the log of the concentration of the inhibitor (T).

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different glycosylation of the toxin molecules (15). As expected, rRTA migrated faster than glycosylated nRTA. cRTA separated instead into two electrophoretically distinct forms, both recognized by anti-RTA antibody. Microsequencing of proteins recovered from SDS-PAGE revealed the presence of a proteolytic cleavage site at Arg30 responsible for the removal of the KFT25 peptide in cRTA molecules (Fig. 1), which accounted for the doublet in lane 3.

To investigate whether cRTA retained the enzymic properties of the original molecule, the protein synthesis inhibition activity of nRTA, rRTA, and cRTA was compared in a rabbit reticulocytelysate. cRTA inhibited protein synthesis in a manner that was comparable to nRTA and rRTA (IC50 5, 26.6, and 40 pM, respectively). These results demonstrated that fusion to KFT25 had not affected the enzymic properties of RTA.

Protein G of the vesicular stomatitis virus, reconstituted in phospholipid vesicles, was shown to induce liposome fusion at pH 5.0 using PC/PS (1:1 molar ratio) SUV as target vesicles (16). The rate of fusion dramatically increased at pH values in the range 2.0–4.0 (16).

Lipid vesicles possess the well defined property of deviating light in a way highly dependent on their dimensions and aggregation state. Hence, the amount of light scattered by the lipid suspension is a very sensitive parameter of phenomena leading to liposome aggregation and/or fusion that may be triggered by the interaction of a protein with the lipid layer (17–19). To directly evaluate the acquired pH-dependent membrane destabilizing properties of cRTA, we have measured the changes in the light scattering shown by a PC/PS (1:1 molar ratio) SUV suspension in the presence of cRTA at different pH values. For comparison, phospholipid vesicles were also treated with nRTA.

Fig. 2 shows the pH dependence of the changes in light scattering of SUV treated with cRTA and nRTA. At pH values below 5.0, the light scattering of SUV increased rapidly following the addition of cRTA (t1/2 ≈ 2 s; Fig. 2A, inset). The kinetics of interaction of nRTA with PC/PS SUV were instead slower (t1/2 ≥ 30 s; Fig. 2B, inset). Even though at early times cRTA had a greater effect than nRTA on PC/PS SUV (Fig. 2A), at later times (stationary state), both toxins induced a comparable increase in the light scattering properties of the SUV suspension (Fig. 2B). These results confirm previous observations that the ricin A chain has intrinsic properties of membrane interaction (20, 21). It is noteworthy that the results shown for cRTA in

![Fig. 1. Cloning and purification of recombinant toxins.](image1)

![Fig. 2. pH dependence of the effects of cRTA and nRTA on PC/PS SUV.](image2)
KFT25 peptide with the toxin sequence.

Fig. 3 shows the dose-dependent pattern of the change in the light scattering of SUV as a function of the amount of cRTA added at pH 3.1. cRTA increased the light scattering of PC/PS SUV at low concentrations, and this increase appeared to reach a plateau at the higher concentrations tested (Fig. 3). At the end of the assays, visible precipitates of phospholipid vesicles were observed, indicating a great extent of vesicle aggregation and/or fusion. It should be noted that cRTA is active on phospholipid vesicles at acidic pH at concentrations comparable to those observed also for diphtheria toxin, whose pH-dependent membrane destabilizing properties are well documented (22).

The membrane destabilizing properties of KFT25 are activated at pH values below 6.0 in erythrocytes and nucleated cells (2, 3). On the other hand, TfN is internalized and transported within endosomes whose pH was shown to be 5.5 (23). To investigate whether cRTA had acquired cytosol localizing properties, we synthesized TfN-cRTA conjugates and compared their cytotoxic effect with that of TfN-nRTA and TfN-rRTA conjugates. SDS-PAGE analysis of the three conjugates under reducing and nonreducing conditions followed by Western blot analysis with anti-TfN and anti-RTA antibodies and scanning densitometry revealed the presence of comparable amounts of nRTA, rRTA, and cRTA conjugated to TfN (TfN/RTA ratios of 1:1.27, 1:1.32, and 1:1.33 for TfN-nRTA, TfN-cRTA, and TfN-rRTA, respectively). To make sure that the conjugation procedures had not inactivated the enzymic properties of nRTA, cRTA, and rRTA, the protein synthesis inhibition activity of the three conjugates was compared in a rabbit reticulocyte lysate. The measured IC50 values were 7, 14, and 10 pm for TfN-nRTA, TfN-cRTA, and TfN-rRTA, respectively, further demonstrating that the three conjugates have comparable enzymic and biochemical properties. It should be noted that these IC50 values are calculated for molecules with Mw values of 118,100, 119,600, and 119,900 for TfN-nRTA, TfN-cRTA, and TfN-rRTA, respectively, and hence, they are not directly comparable with those obtained with unconjugated toxins (see above). Correction of IC50 values obtained with TfN-toxin conjugates for molecular composition revealed that the toxins were not inactivated by the conjugation procedures.

We then investigated the cell killing potential of TfN-nRTA, TfN-cRTA, and TfN-rRTA and of free toxins against tumor cells. As shown in Fig. 4 (upper panel), Jurkat cells were equally intoxicated by unconjugated nRTA and rRTA (IC50 = 1.5 × 106 and 2.2 × 106 EU, respectively), cRTA was instead ~10-fold less toxic against target cells (IC50 = 1.6 × 105 EU) despite its enzymic activity that was comparable to that of nRTA and rRTA (see above). cRTA conjugated to TfN (TfN-cRTA) was, however, 10–20-fold more toxic against tumor cells than TfN-nRTA or TfN-rRTA (IC50 = 1.6, 37, and 20 EU, respectively, in a 24-h protein synthesis inhibition assay) (Fig. 4, center panel). Results comparable to those obtained with Jurkat cells were also observed with Raji, CEM, K562, and MCF7 cell lines (data not shown). Addition of the TfN-RTA IT enhancer monensin increased the cytotoxicity of TfN-cRTA, TfN-nRTA, and TfN-rRTA (IC50 = 1.2, 0.6, and 2.0 EU, respectively, in a 6-h assay) (Fig. 4, lower panel). Monensin also abrogated the differences in cell killing between TfN-cRTA and the other two conjugates. Monensin neutralizes the pH of endocytic vesicles (24). Thus, the higher cytotoxic activity observed for TfN-cRTA in the absence of monensin strongly suggests that the pH-dependent
membrane destabilizing properties of KFT25 might facilitate the translocation of cRTA to the cell cytosol.

The KFT25 peptide contains a potentially reactive Cys residue that could intervene in the disulfide-based linkage of cRTA molecules to Tfn during IT synthesis. To rule out that the greater cytotoxic effect shown by Tfn-cRTA in the absence of monensin could be due to a spacer effect (25), we also created a new chimeric toxin (scRTA) by genetically fusing to the RTA gene an oligonucleotide coding for the the 25-amino acid unrelated peptide Gly-Ser-(Gly)_2-Ser-(Gly)_2-Ser-(Gly)_2-Cys-Pro. The scRTA sequence was then expressed in E. coli, and the purified toxin was conjugated to Tfn following the same experimental procedures as described for cRTA and rRTA. As shown in Fig. 4, un conjugated scRTA as well as Tfn-scRTA displayed cytotoxic activity against Jurkat cells comparably to rRTA and rRTA and their Tfn-toxin conjugates. These results demonstrate that the Cys residue present in the KFT25 peptide is unlikely to play a role in the molecular mechanisms leading to the higher cytotoxic activity of Tfn-cRTA.

Cell intoxication by IT is a multistep process, first involving binding of the IT molecules at the cell surface. It is well known that the cell killing kinetics of the IT are strictly dependent on the affinity of the interaction between the IT and the target receptors (26). To rule out that the higher cytotoxic effect shown by Tfn-cRTA was due to a more efficient binding to target cells, we compared the binding capacity of Tfn-nRTA, Tfn-cRTA, and Tfn-rRTA on Jurkat cells by applying the method described by Schil (14) as modified by Ittelson and Gill (12). This method is based on the inhibition by a specific competitor (i.e. Tfn) of the cytotoxic effects mediated by a cytotoxin (i.e. Tfn-rRTA conjugates). It should be mentioned that this procedure allows the K_I of the competitor and not of the cytoxin to be measured. However, if the competitor inhibits to the same extent the cytotoxic effect of different cytotoxins directed against the same receptor, then it can be concluded that the cytotoxins bind the common receptor with equal affinity. This procedure was chosen because it does not require radioisotope labeling of the molecules involved, thus preventing inactivation of the ligands or alteration of the ligand/receptor interactions. Moreover, the sensitivity of this method is considerable because it is based on the biological activity of enzymic cytotoxins (e.g. RTA). As shown in Fig. 5, binding of Tfn-nRTA, Tfn-cRTA, and Tfn-rRTA to Jurkat cells was comparable. Displacement of Tfn-vehicled toxins by Tfn also demonstrated that the three conjugates bound the transferrin receptor in a specific manner.

Unlike other pharmacological antitumor reagents, IT are effective at very low concentrations both in vitro and in vivo (1). However, the fraction of IT molecules reaching the target cells of a solid tumor is often despairingly low due to a number of physiologic barriers preventing diffusion of the IT within the tumor and drastically impairing their therapeutic efficacy (e.g. high interstitial pressure, low diffusion rates of macromolecules within the tumor, antigen-site barrier, inadequate pharmacokinetics, immune-mediated clearance mechanisms) (27). To enhance IT cytotoxicity in vivo, the combined use of RTA IT and of the carboxylate ionophore monensin has been proposed. However, in vivo application of monensin or of its protein-conjugated derivative human serum albumin-monensin may be problematic due to the monensin inactivating properties of the serum (28–30). An alternative approach involves the possibility of enhancing IT cytotoxicity by directing them to intracellular compartments where translocation of the IT to the cytosol is facilitated. Retention signals have been added to Pseudomonas exotoxin and to RTA to ease their delivery to the endoplasmic reticulum lumen from where Pseudomonas exotoxin and RTA are thought to enter the cytosol (31, 32).
potential might be obtained by using targeting molecules residing for longer times within acidified vesicles following internalization.

By linkage to KFT25, the toxicity of RTA against intact cells has been reduced 10-fold, and therefore, its toxicity toward non-target cells has been concomitantly decreased by the same factor. This might be advantageous because cRTA-based IT would offer a larger therapeutic window with respect to nRTA or rRTA-based IT. Considering the difference in cytotoxicity between conjugated and unconjugated toxins, the “specificity factors” are in fact 100,000, 405, and 1100 for Tfn-cRTA, Tfn-nRTA, and Tfn-rRTA, respectively (Table I).

The interaction of positively charged biomolecules with negatively charged lipid membranes has been implicated in several biological processes. Some examples are membrane permeabilization or perturbation and membrane-membrane aggregation. Basic polypeptides as well as clusters of positively charged residues in several proteins have been shown to have aggregation. Basic polypeptides as well as clusters of positively charged lipid membranes has been implicated in several viral structures to be linked to toxins of different origin.

In conclusion, our results demonstrate that it is possible to exploit the strategies developed by viruses to enter eukaryotic cells in order to enhance the specific cytotoxic effect of IT. A frame is also set for further studies aimed at selecting the most appropriate viral cell structures to be linked to toxins of different origin.

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