Ricin A Chain Can Transport Unfolded Dihydrofolate Reductase into the Cytosol*

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Ricin is a heterodimeric protein toxin. The ricin A chain is able to cross the membrane of intracellular compartments to reach the cytosol where it catalytically inactivates protein synthesis. It is linked via a disulfide bond to the B chain, a galactose-specific lectin, which allows ricin binding at the cell surface and endocytosis. To examine the potential of ricin A to carry proteins into the cytosol and the requirement for unfolding of the passenger protein, we connected mouse dihydrofolate reductase (DHFR) to ricin A by gene fusion via a spacer peptide. DHFR-ricin A expressed in *Escherichia coli* displayed the biological activities of the parent proteins and associated quantitatively with ricin B to form DHFR-ricin. The resulting toxin was highly cytotoxic to cells (4–8-fold less than recombinant ricin). DHFR-ricin cytotoxicity was inhibited by methotrexate, a DHFR inhibitor stabilizing DHFR-ricin A in a folded conformation. The DHFR moiety of DHFR ricin bound to the plasma membrane. Although methotrexate prevented this binding, it did not significantly affect DHFR-ricin endocytosis, which proceeded via ricin B chain. Intoxication kinetics data and a cell-free translocation assay demonstrated that protection of cells from DHFR-ricin cytotoxicity resulted from a selective inhibition by methotrexate of DHFR-ricin A translocation. We conclude that ricin A is a potential carrier of proteins to the cytosol, provided that the passenger protein is able to unfold for transmembrane transport.

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‡ The abbreviations used are: DT, diphertheria toxin; DTA, diphertheria toxin A chain; CTL, cytotoxic T lymphocyte; DHFR, dihydrofolate reductase; MTX, methotrexate; RTA, ricin A chain; RTB, recombinant RTA; RTB, ricin B chain; Pipes, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; PBS, phosphate buffered saline.

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EXPERIMENTAL PROCEDURES

Materials—All enzymes needed for DNA manipulations were obtained from Life Technologies, Inc. or Pharmacia. Total DNA polymerase for polymerase chain reaction, plasmid prep purification system, DNA silver sequencing kit, and reticulocyte lysates were from Promega. Most of the chemicals were obtained from Sigma. Pure RTB (without any detectable RTA) was purchased from Inland Biologicals, Austin, TX, whereas recombinant RTA (rRTA) was provided by Zeneca (UK). Western blotting detection kit and radiochemicals were from Amersham. Whereas recombinant RTA (rRTA) was provided by Zeneca (UK). Western blotting detection kit and radiochemicals were from Amersham. DNA silver sequencing kit, and reticulocyte lysates were from Promega.

Preparation of the DHFR-RTA Chimera—An EcoRI-PstI DHFR fragment was obtained by polymerase chain reaction using the full-length coding sequence of the DHFR gene in pDS55 vector (21) as a template. The sense primer (CTAAG AATTCCATTTGTTGACATTG) was used to introduce an EcoRI site (↓) immediately upstream of the initiation codon. The antisense primer (CTTACTGCA GGTCTTTCTCCTCTG) provided a PstI cleavage site (↓) immediately before the natural stop codon. The full-length RTA-coding sequence (22) in pKK 223.3 (Pharmacia) was used as a template to prepare by polymerase chain reaction an PstI-HindIII RTA fragment. The sense primer (GATCTTCGCA ↓ GATATTCCTCCCAACAACCA) provided a PstI site (↓) before the second codon of RTA and the antisense primer (AGCTTC- AAAACTGTTGACAGCA) provided a HindIII site (↓) after the stop codon. Purification of recombinant DHFR-RTA was achieved by inclusion of a fluorescent His6 epitope and removal of this epitope with a 6xHis affinity tag ligated in a stepwise manner into pKK 223.3. Preliminary experiments showed that the fusion protein prepared without any spacer was inactive in several assays for biological or biochemical activities (data not shown). A double-stranded oligonucleotide containing an internal BamHI site and coding for a spacer peptide (His-Ala-Ser-Thr-Pro-Glu-Pro-Asp-Pro-Val) was thus inserted using the PstI site. This peptide linker is similar to the flexible hinge region of a monoclonal antibody (23). Purified plasmids were restricted with PstI before transformation of E. coli TG2. Clones were screened for acquisition of the BamHI restriction site, and DNA sequencing enabled the assessment of its orientation. The resulting protein is denoted DHFR-RTA.

Expression and Purification of the Fusion Protein—A 1.5 liter culture of E. coli TG2 containing the DHFR-RTA plasmid was grown at 30 °C. Expression was induced at an A550 of 0.4 using 1 mM isopropylthiogalactoside. After 2 h at 30 °C, E. coli lysates were prepared by sonication, clarified by centrifugation for 30 min at 20,000 × g (22), and dialyzed against 20 mM potassium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5 mM diithothreitol (buffer A) before loading onto a 5–30% linear sucrose gradient (Pharmacia). After washing with 45 ml of buffer A and 60 ml of buffer A supplemented with 1 mM KCl, DHFR-RTA was eluted with 1 mM MTX in buffer A and stored sterile at 80 °C, after adding 15% glycerol. The final yield of purified protein was greater than 10 mg/liter of culture. DHFR activity was measured as described previously (24). The ability of RTA to inhibit protein synthesis was assayed using rabbit reticulocyte lysates (25) supplemented with globin mRNA (Life Technologies, Inc.).

Association of DHFR-RTA with RTB; Protein Labeling—The two proteins (20 μl each) were mixed in PBS in the presence of 8 mM GSH. After 3 h at room temperature and overnight dialysis at 4 °C against PBS, the mixture was analyzed by nonreducing SDS-PAGE. Recombinant ricin (rRTA-RTB) was prepared using the same protocol. To examine the kinetics of protein synthesis inactivation by recombinant ricin and DHFR-ricin the above protocol was modified. The cell number was increased to 70,000/well (BW5147) or 15,000/well (L929). A pulse incorporation of [35S]methionine (0.25 μCi) was added, and cells were incubated for a further 12–18 h. Precipitation with trichloroacetic acid was then performed either directly (BW5147) or following solubilization with 0.1 N NaOH after aspiration of the medium (L929). Proteins were collected on Whatman paper filters, washed twice with 5% trichloroacetic acid, then dried for radioactivity determination. Background incorporation was obtained from cells treated with 1 mM cycloheximide.

To examine the kinetics of protein synthesis inactivation by recombinant ricin and DHFR-ricin the above protocol was modified. The cell number was increased to 70,000/well (BW5147) or 15,000/well (L929). A pulse incorporation of [35S]methionine (1 h) was performed at various times after the start of incubation.

Study of DHFR-RTA Stability during Intoxication by DHFR-Ricin—Radiolabeled toxin (5 nmol of [125I]-DHFR-ricin or [125I]-recombinant ricin) was added to BW5147 cells (107/ml of RPMI/fetal calf serum) in the presence or absence of 50 mM MTX. After 0–24 h at 37 °C, cells were collected by centrifugation, washed three times with PBS, then lysed in 1 ml of immunoprecipitation buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 μM phenylmethylsulfonyl fluoride, 1% Nonidet P-40) (30). After 20 min on ice, insoluble material was removed by centrifugation (13,000 × g for 10 min). This step and the rest of the experiment were performed at 4 °C. The cleared lysate received 2 μl of sheep anti-RTA antibody and was mixed for 1 h on a rotating wheel before adding 7 μl of protein G-agarose (Sigma). After 1 h on the wheel, the immune complexes were recovered by centrifugation (10,000 × g for 3 min), washed twice with immunoprecipitation buffer, then once with PBS, and eluted by boiling in SDS-PAGE reducing sample buffer (30). Gels were exposed to storage phosphor screens that were analyzed using a Storm apparatus.

Endotoxins and Cell-free Translocation Assay—Endotoxins efficiency of radiolabeled DHFR-RTA (DHFR-ricin) by mouse BW5147 lymphocytes was measured using recombinant ricin as a control. Washes with 0.1% lactose were used to displace plasma membrane-bound molecules (6, 28).

The assay for toxin translocation from purified lymphocyte endosomes is described elsewhere (6). 125I-Transferrin, as a membrane-bound tracer, and 125I-labeled ricin A chain, as a soluble marker, were used as controls in all translocation experiments to determine the integrity of endosomes. BW5147 cells were labeled with DHFR-ricin for 30 min at 37 °C in Dulbecco’s modified Eagle’s medium containing 0.2 mg/ml bovine serum albumin and 0.15 mg/ml low density lipoproteins/ml, before lactose-scraping to displace membrane-bound ligand, ricin-gold binding, and lysis under hypotonic conditions. Unbroken cells and nuclei were removed by low speed centrifugation, and crude mem-

branes collected by ultracentrifugation. They were then layered on a discontinuous sucrose gradient (40%/30%/20% sucrose). After 2 h at 100,000 × g, endosomes were obtained from the 30%/20% interface, washed, and finally resuspended in translocation buffer (110 mM KCl, 15 mM MgCl2, 1 mM diithothreitol, 0.15 mg/ml bovine serum albumin, 20 mM HEPES, pH 7.1, supplemented with ATP except when otherwise indicated). Translocation was assayed for 2 h at 37 °C and was stopped by chilling on ice. The medium was then separated from endosomes by ultracentrifugation (160,000 × g for 5 min) on a 17% sucrose cushion. Translocated proteins were precipitated with 10% trichloroacetic acid and separated by reducing SDS-PAGE before autoradiography. Quantification was performed by densitometric analysis of films exposed with a linear range of detection. Direct counting of slided gels was occasionally used to follow [125I]-RTA or [125I]-RTB translocation and gave identical results. When translocation of unlabeled DHFR-ricin and recombinant ricin was examined, Western blots (29) of translocated proteins were quantified using a Storm apparatus (Molecular Dynamics).

Confocal Microscopy—Exponentially growing BW5147 cells were washed, then labeled with DHFR-ricin-FITC and ricin-tetramethylrhodamine isothiocyanate for 30 min at 37 °C in Dulbecco’s modified Eagle’s medium/bovine serum albumin/low density lipoprotein (see above). After lactose scraping, cells were fixed for 15 min at 2 °C in PBS containing 3.7% parformaldehyde before quenching for 15 min using 50 mM NH4Cl in PBS. They were then washed with PBS, mounted in PBS supplemented with 2.5% 1,4-diacylbicyclo(2,2,2)octane, and examined using a Leica confocal microscope using a 63 × lens and medial optical sections. Bleed through from one channel to the other was negligible. In preliminary experiments transferrin-FITC and an anti-mouse CD45 (clone I3/2) labeled with Cy5 were used as endosomal and plasma membrane markers, respectively (27).

Cytotoxicity Assays—Cells (15,000 BW5147 in RPMI, 10% fetal calf serum or 6,000 L929 in RPMI, 5% fetal calf serum) were seeded in 96-well tissue culture plates. Toxin solutions were added immediately (BW5147) or after 2 h at 37 °C to allow cell adherence (L929). After 24 h at 37 °C ([35S]methionine (0.25 μCi) was added, and cells were incubated for a further 12–18 h. Precipitation with trichloroacetic acid was then performed either directly (BW5147) or following solubilization with 0.1 N NaOH after aspiration of the medium (L929). Proteins were collected on Whatman paper filters, washed twice with 5% trichloroacetic acid, then dried for radioactivity determination. Background incorporation was obtained from cells treated with 1 mM cycloheximide.

RESULTS

Production and Purification of DHFR-RTA—Both DHFR (18) and RTA (31) have been fused directly with a targeting protein to prepare biologically active hybrids. To obtain a biologically active DHFR-RTA chimera, we found it necessary to insert a linker peptide including three prolines (23) between the N terminus of recombinant RTA and the C terminus of DHFR (data not shown). The fusion protein was expressed in E. coli. A major band corresponding to the expected molecular mass of 50 kDa and reacting to both anti-RTA and anti-DHFR
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Expression of the DHFR-RTA fusion protein in E. coli. Soluble proteins from lysed E. coli TG2 transformed with pKK 223.3-DHFR-RTA were separated under reducing conditions by SDS-PAGE before staining with Coomassie Blue or transfer to nitrocellulose for Western blotting. Lane 1, molecular mass markers (66, 45, 36, 29, 24, 20, and 14 kDa); lanes 2 and 5, noninduced bacteria; lanes 3, 4, 6, bacteria after induction of expression; lane 7, rRTA.

FIG. 1.

The DHFR-RTA fusion was purified to homogeneity by affinity chromatography using immobilized MTX (MTX-agarose), as ascertained by protein staining and Western blotting using antisera against both DHFR and RTA (Fig. 2). In particular, cross-reactive material observed in E. coli sonicates using anti-DHFR sera was eliminated by this single chromatographic step (compare lane 4 in Fig. 1 and lane 3 in Fig. 2). A typical yield was greater than 10 mg/liter of culture.

Since DHFR-RTA was purified using its affinity for MTX, it is clear that the DHFR portion of the chimera recognizes MTX. This binding is known to stabilize the folded conformation of DHFR (9). The high protease resistance of DHFR-RTA, probably due to the well documented resistance of the RTA moiety of the chimera to proteases (13), prevented us from monitoring the folding of the DHFR moiety using protease protection experiments, as conventionally performed (8–10, 18). On the other hand, this resistance conferred intracellular stability on the fusion protein (see below).

After purification, DHFR-RTA was tested on rabbit reticulocyte lysates for its ability to inactivate protein synthesis. The resulting IC<sub>50</sub> (30 ± 10 nM) is similar to that of rRTA (15 ± 7 nM). DHFR-RTA also exhibited a DHFR activity (2.5 ± 10<sup>6</sup> units/mg), which is virtually identical to the catalytic activity of native mouse DHFR (2.7–2.8 ± 10<sup>6</sup> units/mg) (11). Together these data indicate that connection of DHFR to the N terminus of rRTA did not affect enzymatic activity of either of the parent proteins.

Preparation of DHFR-Ricin—Mixing equimolar amounts of the DHFR-RTA with RTB and 8 mM GSH before dialysis generated homogenous DHFR-RTA-RTB (DHFR-ricin) as seen by nonreducing SDS-PAGE (Fig. 3, lane 6). This shows that the chimera, DHFR-RTA, interacts as efficiently as rRTA with the B chain. Formation of RTB dimers is observed only when the A chain is absent from the mixture (lane 4). Recombinant ricin (rRTA-RTB, lane 1) was generated using the same procedure. This toxin was used as a control throughout the rest of this study. Recombinant ricin migrated slightly faster (lane 1) than native ricin (shown in lane 2) due to the absence of oligosaccharides on the A chain.

DHFR-Ricin Is Highly Cytotoxic—To examine the ability of rRTA to bring DHFR in the cytosol we first investigated intoxication of various cell types by DHFR-ricin. The results of these tests are summarized in Table I. The isolated chains of ricin were essentially nontoxic, whereas DHFR-RTA surprisingly showed moderate cytotoxicity, indicating that fused DHFR somehow promotes more efficient RTA uptake. Nevertheless, since association of DHFR-RTA with RTB to produce DHFR-ricin increased toxicity by 150–650-fold (Table I), DHFR-mediated internalization was not significantly involved in DHFR-ricin cytotoxicity as compared with uptake via the B chain (see below). DHFR-ricin is only 4-fold less toxic than ricin to L929 fibroblasts. It is also highly toxic to BW5147 lymphocytes (8- to 10-fold less than ricin).

DHFR-RTA Is Stable within Cells—To examine the possibility that DHFR-ricin toxicity resulted from processing of DHFR-RTA within cells to generate RTA, which would then be free to translocate to the cytosol, we studied the stability of DHFR-RTA within cells during intoxication by DHFR-ricin. As shown in Fig. 4, 125<sup>I</sup>-DHFR-Ricin is stable upon BW5147 cells intoxication by 5 nM 125<sup>I</sup>-DHFR-ricin, and no processing could be observed even after 24 h of contact. Quantitative analysis of images obtained from storage phosphor screens showed that beyond 24 h less 125<sup>I</sup>-DHFR-RTA was recovered from cells (Fig. 4). This likely arose because more than 92% of cells have by then been killed by 5 nM DHFR-ricin and consequently a proportion of cells are nonpelletable by centrifugation.

Identical results have been observed when 125<sup>I</sup>-rRTA was taken up by cells in the form of free subunit or as 125<sup>I</sup>-recombinant ricin (L.M.R., unpublished). These results strongly indicate that, during intoxication by DHFR-ricin, DHFR-RTA

FIG. 2. Purification of DHFR-RTA using MTX-agarose. Clarified sonicates of transformed E. coli were loaded onto an MTX-agarose column and eluted with 1 mM MTX as described in the Materials and Methods. Fractions were analyzed using reducing SDS-PAGE followed by Coomassie Blue staining (15 μg of protein, lane 1), or Western blotting (1 μg of protein) with anti-DHFR (lane 3) or anti-RTA-antiserum (lane 4). Lane 2, molecular mass markers (66, 45, 36, 29, and 24 kDa).

FIG. 3. Formation of recombinant ricin and DHFR-ricin. DHFR-RTA (or rRTA) and RTB (100 nmol each) were mixed with 8 mM GSH in 100 μl of PBS. After 3 h at room temperature and subsequent dialysis, an aliquot of the mixture (18 μg of protein) was separated by nonreducing SDS-PAGE and visualized by Coomassie Blue staining. Lane 1, rRTA + RTB; lane 2, native ricin; lanes 3 and 5, DHFR-RTA; lane 4, RTB; lane 6, DHFR-RTA + RTB.
DHFR-RTA is more concentrated compared with MTX. Nevertheless, most DHFR-ricin molecules were plasma membrane-bound and insensitive to lactose scraping, suggesting that this chimera also interacted with the cell surface via its DHFR moiety. When MTX was present during DHFR-ricin endocytosis, most cell-surface labeling was displaced by the lactose washes, as observed for ricin (Fig. 6). Identical results were obtained using radiolabeled toxins and a conventional (6, 28) internalization assay (not shown). These data show that DHFR-ricin cell surface binding at 37 °C is mediated not only by its RTB (i.e. lactose sensitive), but also by its DHFR moiety (i.e. MTX sensitive). The ability of MTX to prevent this DHFR-mediated binding suggests that this interaction normally requires unfolding of the fused DHFR.

Does MTX affect internalization of DHFR-ricin? Neither confocal microscopic examination (Fig. 6), nor endosome fractionation (not shown) provided any evidence for this. We conclude that DHFR-ricin binding via DHFR is not significantly involved in its internalization, which is largely directed by RTB in a process which is insensitive to MTX. These observations are in complete agreement with the cytotoxicity data (Table I).

Translocation of DHFR-Ricin in Intact Cells Is Inhibited by MTX—We first examined the action of MTX on the translocation rate of DHFR-RTA using cytotoxicity assays. Previous studies have shown that the kinetic profile of protein synthesis inactivation by ricin consists of an initial lag followed by a (pseudo)first order decrease in protein synthesis. This slope directly reflects the membrane translocation rate of RTA (5, 32). Using BW5147 lymphocytes, a concentration-dependent lag was observed before the onset of protein synthesis inacti-
RTA translocation is significantly inhibited by MTX. Similar results were obtained using L929 cells (data not shown).

To study more selectively the inhibition of DHFR-RTA translocation by MTX we made use of the only cell-free system available to follow ricin translocation across intracellular membranes. This assay uses endosomes purified from lymphocytes, enabling to follow ricin translocation by MTX—plus (closed symbols) or minus (open symbols) 50 nM MTX, and then pulsed for 1 h with [35S]methionine to measure protein synthesis. Plots of log protein synthesis versus the incubation time plus one-half the [35S]methionine pulse time. When MTX was used, the data were corrected by deducting the inhibitory effect of MTX on cell protein synthesis (a 30–40% inhibition was caused by 50 nM MTX after 22 h). A, 30 pm (○) or 100 pm (●) recombinant ricin; B, 3 nM DHFR-ricin.

Cell-free Translocation of DHFR-Ricin Is Blocked by MTX—To study more selectively the inhibition of DHFR-RTA translocation by MTX we made use of the only cell-free system enabling to follow ricin translocation across intracellular membranes. This assay uses endosomes purified from lymphocytes labeled with [125I]-ricin (6). As reported earlier for native ricin (6), when translocation of radiolabeled recombinant ricin was examined, both [125I]-rRTA (30 kDa) and [125I]-RTB (34 kDa) were transported through the endosome membrane (Fig. 8A). Background [125I]-material present in the medium at the beginning of the experiment was released during the last homogenization step. Both ricin chains displayed the same translocation rate whether translocation of recombinant (Fig. 8A) or native (6) ricin was examined, demonstrating that rRTA behaves like native RTA in this assay. Negative controls such as [125I]-transferrin, a membrane-bound ligand, and [125I]-horseradish peroxidase, a soluble tracer, were not transferred through the endosome membrane (6).

Translocation of [125I]-DHFR-ricin proceeded at half the rate of [125I]-recombinant ricin (not shown) indicating that [125I]-DHFR-RTA translocation is below 10–15% of the efficiency of [125I]-rRTA translocation. Most of the material transported through the membrane of [125I]-DHFR-ricin-loaded endosomes was thus [125I]-RTB whose translocation rate was unaffected whether [125I]-RTB was endocyotised with [125I]-rRTA or [125I]-DHFR-RTA (not shown). These data are in agreement with our previous results demonstrating independent translocation of ricin chains (6).

The translocation rate of radiolabeled recombinant ricin was unaffected when as much as 10 μM MTX was added to the media (shown in Fig. 8B for [125I]-RTA). When the same experiment was performed with [125I]-DHFR-ricin-loaded endosomes, a complete inhibition of [125I]-DHFR-RTA translocation by MTX was observed (Fig. 8B), whereas [125I]-RTB translocation remained unchanged (not shown). These results indicate that, although the ability of MTX to cross biological membranes can be questioned (8), enough MTX molecules could enter the endosome lumen to affect DHFR-RTA transmembrane transport when 10 μM MTX was used in the cell-free assay. Translocation of DHFR-RTA was also selectively blocked when DHFR-ricin endocytosis and all subsequent steps were performed in the presence of 1 μM MTX (a concentration not affecting uptake over the labeling time) to ensure maximum endosome loading by MTX (not shown). The RNA N-glycosidase activities of DHFR-RTA and RTA, as checked on rabbit reticulocyte lysates, were insensitive to MTX up to 10 μM (not shown).

Altogether, these data provide compelling evidence that protection of cells by MTX from DHFR-ricin toxicity arises by selectively inhibiting the translocation of DHFR-RTA.

**DISCUSSION**

Unfolding is a general requirement for protein translocation into the ER (10), the mitochondria (9), or through E. coli plasma membrane (8). Appropriate targeting sequences fused to the N terminus of mouse DHFR has enabled translocation in these systems. Binding of the folate analogue MTX to the DHFR portion of these fusions prevents their unfolding and consequently their transport through the target membrane. The presence of NADPH (a cofactor of DHFR) was required in addition to MTX to block the translocation of a fusion protein consisting of DHFR connected to the precursor of outer mem-
brane protein A (8), although this cofactor is not always essential.

Previous studies on DTA (14) and rRTA (15) translocation where the toxins were internally cross-linked by disulfide-bridge engineering indicated that their unfolding was a prerequisite for translocation. A similar conclusion was reached using fusion proteins between DTA and the acidic fibroblast growth factor (17) or DHFR (18): a specific ligand impairing folding of the transported protein prevented translocation of the chimera.

There are a number of known differences between the translocation mechanisms of DTA and RTA (1, 2). One of these is the energy source, namely, direct ATP hydrolysis for RTA and the pH gradient (endosome-cytosol) for DTA (2, 5, 6). A further difference is the requirement for B chain to assist directly in the membrane traversal step. DTA translocation depends on the presence of the DT B chain-translocation domain, whereas for ricin, RTB can be readily replaced by other ligands (1). It was not therefore obvious that the requirement for unfolding in the case of DTA passenger proteins would also be relevant in the case of RTA.

Here we report the first successful use of RTA to transport a protein through membranes. DHFR-RTA, a fusion protein consisting of RTA fused via a spacer to the C terminus of DHFR, was produced while maintaining the biological activity of the parent proteins: both catalytic activities were apparent, and the propensity to associate with RTB remained unperturbed. The converse fusion, RTA-DHFR was produced in E. coli, but was unable to associate with RTB (data not shown). This was probably due to steric hindrance of the RTA C terminus which is normally involved in a number of non covalent interactions with RTB (33).

Using DHFR-ricin, a strong inhibition of membrane translocation by MTX was observed when studying inactivation curves of protein synthesis by intact cells. Unfortunately, the cytotoxicity of MTX alone limited the maximum concentration which could be used for these experiments to just 50 nM MTX. A complete arrest of DHFR-RTA translocation could be obtained using a cell-free ricin-translocation assay and micromolar MTX concentrations. These data indicate that unfolding of the passenger protein is required for translocation.

DHFR fusion proteins have been invaluable tools to study protein import into mitochondria (9, 34), and DHFR-RTA was therefore selected to provide information on RTA translocation intermediates. An unexpected observation from this study was the affinity of the ricin-fused DHFR for the cell surface in the absence of MTX. Such binding of DHFR has never before been reported. For most translocation studies, DHFR was targeted to intracellular organelles (8–11) and DHFR-DT binding to entire cells was examined at room temperature for 20 min (18), conditions unlikely to reveal interaction of fused DHFR with the plasma membrane, a process which requires over 10 min at 37 °C to become significant (not shown).

The ability of MTX to impair binding of ricin-fused DHFR to the cell surface stresses the point that drugs can affect the way cells handle toxin-conjugates at several stages. It is therefore necessary to separately examine all the steps involved in cell intoxication. Here, MTX was found to inhibit both the translocation of DHFR-RTA and the initial cell-surface binding of DHFR-ricin. Nevertheless, only the first of these MTX effects resulted in protection from DHFR-ricin. Furthermore, MTX did not affect cell-surface binding via RTB, which appears to be the only ligand allowing significant endocytosis of DHFR-ricin and subsequent cytotoxicity.

Truncated Pseudomonas exotoxin A efficiently introduced cellular viral epitopes in the cytosol of target cells for sensitization and lysis by peptide-specific CTLs (16). On the basis of our findings, enzymatically inactive rRTA would appear to be a suitable candidate to transport antigens into the cytosol for stimulation of cell-mediated immunity (19). A double codon mutation ensures virtually complete (i.e. > 1,000-fold) reduction in catalytic activity of RTA (35).

The CTL epitope (usually less than 20 residue long) could be envisaged as a fusion at the N terminus of this RTA mutant. It is known that the N-terminal residue is the only identified targeting signal for degradation via the ubiquitin-proteasome pathway. This cytosolic processing is required for subsequent transport of the epitope to the endoplasmic reticulum lumen followed by presentation at the cell surface by major histocompatibility complex class I molecules and activation of CD8+ CTLs (20). From the ability of DHFR-RTA to interact with RTB, an enzymatically-inactive rRTA with a short N-terminal extension should readily associate with RTB to ensure efficient endocytosis prior to delivery of the CTL epitope to the cytosol. The potential superiority of ricin over bacterial toxins for antigen delivery to the cytosol and CTL induction due to its higher number of cell surface binding sites (1) remains to be tested.

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