ATP-dependent Translocation of Ricin across the Membrane of Purified Endosomes*

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Ricin translocation was demonstrated (using both fluorescence- and radiolabel-based assays) across the membrane of endosomes purified from mouse lymphocytes. Selectivity of the process was shown by the absence of translocation activity of transferrin and horseradish peroxidase used as membrane-bound and fluid-phase endosome labels, respectively. Endocytosed 125I-rinic translocation was found to be strictly ATP- (K_m ≈ 4 mM) and temperature-dependent, with up to 30% endosomal 125I-rinic appearing in the external medium after 2 h at 37 °C. No treatments neutralizing the acidic endosome pH (ammonium chloride, nigericin, chloroquine) significantly impaired ricin translocation, and the pH gradient across the endosome membrane is not required for this process. Chase experiments showed that the ability of 125I-rinic to translocate increases with its depth in the endocytic system (i.e., plasma membrane ≪ early endosomes ≪ late endosomes). Both A and B ricin chains displayed translocation ability as demonstrated by the results of our assay on ricin, ricin B, transferrin-rinic A, and transferrin-rinic B conjugates. Biological activity of both ricin chains is preserved after translocation as shown by the inhibitory effect of the A chain on cell-free protein synthesis and the binding of the B chain to lactose-agarose.

Ricin and diphtheria toxin (DT) are powerful cellular poisons since a single molecule of these proteins is believed to be capable of killing a cell (1, 2). These toxins are made of polypeptide chains A and B. The B chain enables binding at the plasma membrane via recently identified receptors for DT (3) and galactose residues for ricin (1, 4, 5). The A chain is an enzyme which, once in the cytosol, inactivates protein synthesis thus inducing cell death (1-6).

The process of cell intoxication by DT is well known. After endocytosis of this toxin, the low pH (5-5.5) encountered in the endosome induces conformational changes resulting in the selective transfer of the A chain through the endosome membrane (1, 6, 7). The intracellular pathway used by ricin to kill cells is less well documented. After endocytosis via both clathrin-dependent and clathrin-independent pathways, ricin also enters the endosome (1, 2, 4, 5, 8-11). The A chain can already undergo some translocation at this membrane boundary to reach the cytosol (9-11). Some endosomal ricin is routed to the trans-Golgi network (TGN) which seems to be the favorite organelle in the endocytic pathway for ricin A chain translocation (1, 2, 4, 5). Hence, in contrast to DT, late endocytic structures seem to be involved in ricin A transfer to the cytosol.

In brefeldin A (BFA)-treated cells, ricin can still be observed in the TGN (12) which, albeit morphologically altered, remains functional according to receptor endocytosis and recycling (13, 14). The rest of the Golgi apparatus is disconnected from the TGN in the presence of BFA (12-14), and ricin cytotoxicity is inhibited in these conditions (12, 15, 16). Hence, Golgi integrity seems to be necessary for cell intoxication by ricin. A likely hypothesis is that ricin translocation requires a cellular component whose synthesis is blocked by BFA (15). This agrees with the absence of any protective effect of BFA against the cytotoxic effects of immunotoxins prepared by linking the ricin A chain to a monoclonal antibody and which would not need this ricin "receptor" (15).

Further data support the notion of intracellular receptors for ricin (17, 18), and the respective involvement of all components of the endocytic pathway in the ricin intoxication process remains to be established. Nevertheless, the mechanisms of ricin A escape from all of these compartments probably have some similarities. Study of the translocation step in the intoxication procedure is of special importance because such transport is rate-limiting for ricin cytotoxicity (19).

In this paper we describe reconstitution of the process in a cell-free system using purified endosomes. This assay was already shown to be useful in studying DT translocation (7).

Both ricin chains displayed translocation ability. Ricin was found to be translocated by an ATP- and temperature-dependent mechanism that efficiency increases with the distance traveled along the endocytic pathway.

EXPERIMENTAL PROCEDURES

Chemicals—Except when otherwise indicated, all chemicals were purchased from Sigma (U. K. or France). Ricin was also obtained from castor beans (Baker Seeds Ltd., Sleaford, Lincolnshire, U.K.) using established procedures (20). Ricin A (immunopurified on anti-
B antibodies) and B chains were provided by Dr. P. Gros and Dr. H. Vidal (Sanofi Recherche, Montpellier, France). DT was purified from a crude preparation obtained from the Institut Merieux (Marcy l'Etoile, France) (7).

Conjugate Synthesis and Purification—All transferrin conjugates were prepared using SPDP as cross-linking agent (21).

Transferrin-ricin A (TF-RiA) was prepared as described (22, 23).

We and others2 did not find any cysteines available for conjugation on the isolated B chain which was thus activated, as ricin, using SPDP.

The B chain (7 mg) or ricin (12 mg) was treated in PBS (137 mM NaCl, 2.7 mM KCl, 2.6 mM KH2PO4, 8.8 mM NaH2PO4, pH 7.4) using a 7-fold and 10-fold molar excess of SPDP, respectively. After removing the unreacted SPDP by running on a Bio-Gel P-10 (Bio-Rad) column equilibrated with a 0.1 M acetate 0.1 M NaCl buffer, pH 4.5, dithiothreitol was added up to 50 mM and, after 30 min at room temperature, the derivatized proteins were passed through a T-10 column equilibrated with PBS. After concentration to 1.5 ml using an Amicon microconcentrator (YM-10 membrane), they were reacted with the activated transferrin (23) using a 2.5- and 3-fold excess of ricin B chain and ricin, respectively.

After conjugation for 1 night at room temperature, the protein mixture was concentrated to 80-200 pl (1 ml × 1.5 cm) equilibrated with PBS for TF-RiA or PBS containing 0.1 M lactose (PBS/lactose) for the other conjugates. Analysis of fractions using SDS-PAGE showed that the transferrin conjugates were largely contaminated (20-60% according to Coomassie Blue staining) with unreacted B chain (as reported in this study using TF-RiA (22)) but were free of unreacted ricin B chain (see below, section "Results").

The TF-RiA conjugate was then purified on Blue Sepharose CL-6B (Pharmacia LKB Biotechnology Inc.) (24), whereas TF-RiB and TF-ricin were purified on a lactose-agarose column (Sigma, 2 ml bed volume). For this purpose, these conjugates were applied in 1 ml of PBS to the column at a flow rate of 4 ml/h. After washing with 15 ml of PBS, they were eluted with PBS/lactose.

Transferrin-herosporadich peroxidase and ricin-gold conjugates were synthesized as described previously (25, 26).

Other Methods—Protein assays and SDS-PAGE (6-15% acrylamide gels) were performed as described (25).

Radioactive Translocation Assay—This assay was described previously (7).

RESULTS

Ricin Is Processed through Endocytic Elements Involved in Transferrin Processing—To use transferrin as a control for ricin translocation studies it was necessary to establish that ricin was contained in transferrin-positive endocytic elements, following cell labeling in the conditions used to prepare endosomes. When mouse T lymphoma cells (BW 5147) were incubated with TF-herosporadich peroxidase and ricin-gold for 40 min at 37°C, ricin-gold was always observed in transferrin-positive compartments, in whole cells (Fig. 1a) and in the isolated membranes of all endocytic compartments in which ricin is found. Since ricin translocation studies it was necessary to establish that the label bound to vesicles presenting the original membrane orientation was scraped off using the following procedure: adding lactose up to 0.1 M, followed by warming for 2 min at 37°C, cooling to 2°C, adding trypsin (0.3 mg/ml final concentration), leaving for 20 min at 2°C, and then washing the trypsin by the addition of soybean trypsin inhibitor (0.3 mg/ml final concentration). Membranes were then spun for 30 min at 140,000 × g through a cushion of 20% sucrose before the translocation assay. This lactose/trypsin treatment proved to be efficient on intact cells to displace all plasma membrane-bound label (30). This procedure thus seemed to remove the label from outside-out vesicles efficiently (see "Results").

Fluorescence Translocation Assay—Total volume per assay was 100 ml including or not 10 mM ATP + 10 mM MgCl2. After various periods of time at 37°C, tubes were incubated on ice, and 1.25 ml of 110 mM KCl, 15 mM MgCl2, 50 mM Hepes, pH 8, was added to adjust external pH to 8 just before fluorescence measurements were performed (30). They were corrected using values obtained from an endosome preparation devoid of fluorescent label.

Assay of Biological Activity of Ricin Chains—The enzymatic activity of the transfected ricin A chain was tested on rabbit reticulocyte lysate (22, 23). The activity of the ricin A chain was tested on rabbit reticulocyte lysate (22, 23). Protein synthesis was performed for 1 h at 37°C, tubes were chilled on ice, and 1.25 ml of 110 mM KCl, 15 mM MgCl2, 50 mM Hepes, pH 8, was added to adjust external pH to 8 just before fluorescence measurements were performed (30). They were corrected using values obtained from an endosome preparation devoid of fluorescent label.

Endosome Labeling and Isolation—Mouse T lymphocytes BW 5147 were grown as described (25). The cells at 2 × 10^6/ml in DMEM supplemented with 0.1 mg/ml BSA (DMEM/BSA) and 0.15 mg/ml low density lipoproteins (25) were labeled with a transferrin or ricin derivative for 40 min at 37°C. Fluorescent proteins, TF-herosporadich peroxidase and ricin (or TF-RiA or TF-ricin conjugates), were added as a carrier (70 μl to 1 ml of medium for 10 min at 30°C).Cells were then cooled to 2°C and washed twice with DMEM/BSA supplemented with 0.1 M lactose and with a 2-min incubation at 37°C during the second wash for all ricin B chain-containing label) then with PBS (after a wash with PBS/lactose for ricin derivatives). In preliminary experiments, "lactose scraping" was found to displace all of the 2°C bound 125I-ricin (data not shown), so that only endosomal ricin was left. Since the transferrin-ricin conjugate used in this study displayed no translocation activity at the plasma membrane level (see "Results"), the ricin-gold treatment performed in the original procedure of endosome isolation (25) to eliminate any plasma membrane contamination was omitted.

The cells were then lysed and endosomes isolated. They were finally resuspended in translocation buffer (110 mM KCl, 15 mM MgCl2, 1 mg/ml penicillin G, 1 mM dithiothreitol, 20 mM Pipes, pH 7.1).

Plasma Membrane Labeling and Preparation—For experiments involving the translocation assay at the plasma membrane level, the cells were first labeled with the 125I-conjugate for 1 h at 2°C (equilibrium binding) and thus reached for all conjugates; data not shown). After washing off excess ligand, plasma membrane vesicles were prepared using the same lysis protocol and gradient preparation as that described for endosomes (25). Interfaces between the 40/30% and 30/20% sucrose solutions were collected at the end of the gradient. This allowed elimination of most lysosomes and mitochondria, which stay at the bottom of the 40% sucrose solution (25). Membranes were finally resuspended in 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 8, and the bound label to vesicles presenting the original membrane orientation was scraped off using the following procedure: adding lactose up to 0.1 M, followed by warming for 2 min at 37°C, cooling to 2°C, adding trypsin (0.3 mg/ml final concentration), leaving for 20 min at 2°C, and then washing the trypsin by the addition of soybean trypsin inhibitor (0.3 mg/ml final concentration). Membranes were then spun for 30 min at 140,000 × g through a cushion of 20% sucrose before the translocation assay. This lactose/trypsin treatment proved to be efficient on intact cells to displace all plasma membrane-bound label (30). This procedure thus seemed to remove the label from outside-out vesicles efficiently (see "Results").

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Radioactive Translocation Assay—This assay was described previously (7).

2 P. Gros and Dr. H. Vidal, personal communication.
FIG. 1. Ricin-gold is internalized in transferrin-positive compartments by BW 5147 cells. BW 5147 mouse lymphocytes were incubated for 30 min at 37 °C with Tf-horseradish peroxidase and ricin-gold. Whole cells (panel a) and isolated endosomes (panel b), obtained as described under "Experimental Procedures," were then prepared for electron microscopy observation (25). Bar = 0.1 μm.

FIG. 2. SDS-PAGE analysis of ricin-transferrin conjugates. SDS-PAGE was performed on a 6-15% acrylamide gel and proteins were stained with Coomassie Blue. Reducing gels: lane 1, ricin A chain; lane 2, ricin B chain. Nonreducing gels: lane 3, Tf-ricin; lane 4, Tf-Rib; lane 5, Tf-Ria; lane 6, Tf-Ria before the Blue-Sepharose step; lane 7, transferrin. Molecular masses are indicated in kDa.

separately the translocation ability of each ricin chain and to target them selectively in the endosome compartment. The purity of the hybrids (prepared as described under "Experimental Procedures") is displayed in Fig. 2. The affinity purification step assured that all conjugates were pure (mostly 1:1 hybrids). They were free of unconjugated ricin chain and transferrin.

Fluorescence Translocation Assay—Upon incubation at 37 °C of BW 5147 endosomes loaded with Ri-FI as detailed under "Experimental Procedures," a time-dependent linear increase in the fluorescence ratio 495 nm/450 nm (excitation wavelengths) was observed in the presence of ATP (Fig. 3). Since this increase in the 495/450 fluorescence ratio enabled us to calculate a mean pH of 5.3 for Ri-Fl in the endosome preparation. The same value is obtained for Tf-Fl (25). This is consistent with the endosomal localization of ricin in the preparation.

Radiolabel Translocation Assay—To demonstrate further that ricin (at least one chain) was translocated in this system, i.e. liberated in the medium and not simply exposed to it, we used a radiolabel assay (7) to assess ricin translocation. Upon incubation at 37 °C in the presence of ATP, 125I-ricin-loaded endosomes gradually released label into the surrounding medium. This is shown in Fig. 4, where a loss of 125I-ricin in the pellet (membrane-bound) along with a concomitant increase in the 160,000 × g supernatant (soluble) is apparent. 15% of total membrane-bound 125I-ricin became soluble within 3 h. In subsequent experiments, we routinely found a 30-65% increase in the supernatant/pellet ratio (i.e. a release of up to 30% of endosomal 125I-ricin) in 2 h.

No displacement of 125I-transferrin (the label that was used was ATP-dependent. The observed stability of the Tf-Fl fluorescence level suggests that the integrity of the endosome membrane was retained under these conditions (Fig. 3). The 495/450 fluorescence ratio enabled us to calculate a mean pH of 5.3 for Ri-Fl in the endosome preparation. The same value is obtained for Tf-Fl (25). This is consistent with the endosomal localization of ricin in the preparation.

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FIG. 4. Translocation of \(^{125}\text{I}\)-ricin across the membrane of purified endosomes. After different times at 37 °C, endosomes, prepared from cells labeled with either \(^{125}\text{I}\)-ricin or \(^{125}\text{I}\)-transferrin (and also with ricin if indicated), were separated from the translocation medium by ultracentrifugation (7), and both were counted. The radioactivity ratio (supernatant/pellet) was used to monitor directly translocation, which was assayed in the presence (closed symbols) or absence (open symbols) of 10 mM ATP + 10 mM MgCl\(_2\). Mean ± S.E. (n = 4; error bars are contained within the symbol). ○ and □, \(^{125}\text{I}\)-ricin; ○ and □, \(^{125}\text{I}\)-transferrin; △, \(^{125}\text{I}\)-transferrin with cointernalized ricin (50 μg/ml during cell labeling).

FIG. 5. Temperature dependence of cell-free \(^{125}\text{I}\)-ricin and \(^{125}\text{I}\)-DT translocation. Translocation was assayed in the presence of ATP (7). Control values were obtained from tubes kept on ice, and 100% translocation data refer to the 37 °C value. Mean ± S.E. (n = 4).

FIG. 6. Energy dependence of \(^{125}\text{I}\)-ricin translocation. The indicated nucleotide replaced ATP during \(^{125}\text{I}\)-ricin translocation experiments, performed as described in the legend of Fig. 4. Mean ± S.E. (n = 6). AMPPNP, 5' -adenylyl-P,γ-imidodiphosphate.

Ricin Translocation across Endosome Membrane

regularly as a control throughout this study) from the membrane to the soluble fraction was observed over time at 37 °C, with or without ATP or cointernalized ricin, as shown in Fig. 4.

The use of \(^{125}\text{I}\)-horseradish peroxidase as control gave results similar to those with \(^{125}\text{I}\)-transferrin (see Fig. 8). Nevertheless, cell labeling using \(^{125}\text{I}\)-peroxidase resulted in a radiolabeling level representing less than 0.1% of that obtained at equivalent \(^{125}\text{I}\)-transferrin activity. Hence, because of the very low efficiency of fluid phase endocytosis in this cell type, transferrin was considered to be a more suitable endosome label for this study.

In the absence of ATP, \(^{125}\text{I}\)-ricin translocation reached a plateau after 30 min, whereas translocation was linear for up to 3 h in the presence of ATP (Fig. 4). The better quality signal obtained with this radiolabel technique allowed us to study the requirements for ricin translocation further. All of these experiments were performed using a 2-h incubation time.

Ricin Is Translocating from Endosomes—In this study, we used a well characterized endosome fraction to investigate ricin translocation in a cell-free system. The fraction includes a variety of endosomal elements and is highly enriched in compartments involved in transferrin processing. Lysosomes are not included (25), and TGN elements were barely detected by sialyltransferase activity determination (<0.7% of starting homogenate activity compared with 40% of endocytosed \(^{125}\text{I}\)-transferrin or \(^{125}\text{I}\)-ricin recovery). A few contaminants, i.e. endoplasmic reticulum- and Golgi-derived vesicles, are present, but less than 6% of these cell organelles are recovered in the endosome preparation (25). Hence, since ricin was found in transferrin-positive compartments in this preparation (Fig. 1) and the observed translocation was so active (up to 30% release of \(^{125}\text{I}\)-ricin originally present in isolated endosomes), we can rule out the possibility that these organelles are suppliers of significant amounts of translocated ricin.

Temperature Is a More Critical Factor for Ricin Than for DT Translocation—Fig. 5 shows that ricin translocation is clearly temperature-dependent and hardly detectable below 22 °C. On the other hand, DT translocation is almost 40% as active in the 15–20 °C range as it is at 37 °C. Consequently, translocation is at least one of the steps responsible for the difference in cell sensitivity toward these toxins at temperatures below 20 °C: partly resistant to ricin but still DT-sensitive (1, 2).

ATP Dependence of \(^{125}\text{I}\)-Ricin Translocation—Translocation of \(^{125}\text{I}\)-ricin is strictly ATP-dependent; Fig. 6 shows that none of the nucleotides tested was more than 40% as efficient as ATP in supporting translocation. Although ATP dependence of ricin translocation had been indirectly determined in whole cells using inhibitors (32), there has been no previous direct evidence. We determined a K\(_c\) of 4 ± 2 mM for ATP, similar to the ATP concentration found in lymphocytes (33), thus the ATP requirements for significant ricin translocation are likely fulfilled within the cell.

The Endosome-Cytosol pH Gradient Is Not Required for Ricin Translocation—Ammonium chloride + methylamine (20 mM each), chloroquine (20 μM), and nigericin (5 μM) were all found to equilibrate the pH of isolated endosomes at the buffer level (using either Tf-F1 or Ri-F1 as probe; data not shown). Inclusion of one of these agents in the translocation assay did not alter the \(^{125}\text{I}\)-ricin translocation rate, thus showing that the endosome → medium pH gradient (5.3 → 7.1) is not required for the process (Table I). An identical result was obtained when the same experiment was performed at a different pH (in the pH 5–8 range; data not shown), indicating that ricin translocation is not pH-dependent within this range.

Translocation of \(^{125}\text{I}\)-Ricin Is More Active from Late than from Early Endosomes—As shown in Fig. 7a the longer the incubation of cells with \(^{125}\text{I}\)-ricin before endosome isolation, the more efficiently it was translocated. Moreover, when cells
were allowed to endocytose 125I-ricin for 10 min and then incubated in lactose-containing medium, the translocation efficiency increased with the length of chase (up to 30 min; Fig. 7b). This suggests that within intact cells 125I-ricin translocation preferentially occurs from later endosomal compartments.  

Ricin Is Able to Translocate from Plasma Membrane Vesicles—Since in internalization experiments (and others (8)) found that around 80% of 125I-ricin is plasma membrane-bound after incubation at 37 °C for 30-40 min, it was of interest to examine the ricin translocation potential at this membrane boundary. We used plasma membrane vesicles obtained by homogenization of BW cells prelabeled with 125I-ricin at 2 °C. These vesicles were treated with lactose to remove label bound to cell surface side-out vesicles, and the ability of ricin to translocate from cell surface side-in vesicles was then measured. The results presented in Fig. 8 show that 125I-ricin was able to translocate from these vesicles. This transport of 125I-ricin into the medium during these experiments showed a strict requirement for ATP (Fig. 8), was linearly time-dependent up to 3 h (data not shown), and is therefore not derived from residual label bound to the external side of the vesicles. Nevertheless, translocation from plasma membrane vesicles occurred at a reduced rate (10 ± 5%) relative to that observed with 125I-ricin-labeled endosomes.

Fig. 8 also shows that translocation of control proteins (transferrin, Tf-horseradish peroxidase, horseradish peroxidase) was never observed from either endosomes or plasma membrane vesicles in our assay conditions. Data obtained using 125I-horseradish peroxidase were poorly reproducible because of the low level of endosome labeling with this probe.  

Ricin B Chain Can Cross the Endosome Membrane—The translocation ability of the ricin B chain was first examined using 125I-ricin B-labeled endosomes. This process was ATP-dependent and almost as active as 125I-ricin translocation (Fig. 8).

Intracellular Routing of Transferrin-Ricin Conjugates—To examine separately the potential of each ricin chain to cross the endosome membrane, we synthesized transferrin conjugates to localize them selectively inside endosomes. The endocytosis efficiency (after 40 min at 37 °C) of all hybrids (Tf-RiA, Tf-RiB, Tf-ricin) was around 45%, expressed as the percentage of 125I-labeled molecules remaining after washing the plasma membrane-bound label (using the procedure described under “Experimental Procedures”). This value was < 5% in the presence of excess (1,000-fold) free transferrin, indicating that the transferrin part of the hybrids was directly internalized (data not shown), as shown earlier for a Tf-DT conjugate (7). Hence, the difference in the behavior of these conjugates in the translocation assay compared with Tf-horseradish peroxidase (used as control) could not be caused by a different localization in the endocytic elements.

The Use of Transferrin-Ricin Conjugates Demonstrates That Both Ricin Chains Show Independent Translocation Ability—Although no significant translocation of conjugates was detected at the plasma membrane, they were all found to translocate across the membrane of purified endosomes, as shown in Fig. 8. This process was found to be ATP-dependent for all conjugates. Similar results were obtained for ricin B-containing hybrids when intracellular routing was determined only via the transferrin part of the conjugate (by the addition of 0.1 M lactose during cell labeling; data not shown). It would be difficult to draw any conclusion from the differences in translocation rates between the hybrids since the conjugation procedure with transferrin leads to heterogeneous products.

When translocated proteins were separated by SDS-PAGE (Fig. 9), there was a selective time-dependent release of the ricin A chain and ricin B chain into the supernatant of 125I-Tf-RiA (Fig. 9a) and 125I-Tf-RiB (Fig. 9b) loaded endosomes, respectively. This was confirmed by quantitative analysis of
FIG. 9. Selective translocation of ricin A and ricin B chains from transferrin-ricin conjugates. $^{125}$I-Labeled proteins released from $^{125}$I-Tf-RiA- (panel a), $^{125}$I-Tf-RiB- (panel b), or $^{125}$I-Tf-horseradish peroxidase (HRP)-labeled endosomes during the translocation assay were separated by nonreducing SDS-PAGE after trichloroacetic acid precipitation. Bands were localized by autoradiography (panels a and b, top parts). They were then excised from duplicate gels for radioactivity measurements, the results of which are presented on the bottom parts of the panels. Molecular masses are indicated in kDa.

duplicate gels (Fig. 9), demonstrating that only the ricin subunit of the conjugate was translocated, whereas amounts of the other proteins present as background in the endosome medium did not increase over time at 37 °C. The graphs presented in Fig. 9 were obtained using direct radioactivity determination. Densitometric scanning of autoradiographs provided similar results. The $^{125}$I-A chain appears as one poorly resolved band on these nonreducing gels (Fig. 9a). Background proteins were already present in the medium at the beginning of the assay, suggesting that they had been liberated from endosomes during the last homogenization step, as reported earlier (7). There was no significant translocation of any $^{125}$I-protein from $^{125}$I-Tf-horseradish peroxidase-labeled endosomes used as controls (Fig. 9c).

Both Ricin Chains Are Transported through the Endosome Membrane during Ricin Translocation—To identify which proteins are transferred through the membrane during ricin translocation, the translocated chains have to be separated. This cannot be done by SDS-PAGE (Fig. 2). We used the lectin properties of the B chain to isolate it on lactose-agarose. This was possible since this activity is preserved after translocation; more than 85% of translocated $^{125}$I-ricin B could bind to the gel using a binding assay that involves prior reduction of the sample (see “Experimental Procedures”). In the presence of excess cold A chain, less than 10% of translocated $^{125}$I-ricin A (from $^{125}$I-Tf-RiA-loaded endosomes) bound to lactose in these conditions.

This lactose binding procedure can thus be used to isolate B chains in the medium from ricin translocation assays. Fig. 10 shows that both ricin chains were released at approxi-
mately the same rate from \(^{125}\)I-ricin-labeled endosomes. Hence, the B chain always showed translocation ability whether it was present in the endosome alone (Fig. 8) or conjugated to either transferrin (Figs. 8 and 9) or ricin A (Fig. 10).

It was not possible to obtain any reliable data in the lactose-agarose binding test (or in the protein synthesis inhibition assay) without prior reduction of the translocation medium; the amount of free ricin A chain in the un-reduced translocation medium reached a plateau or decreased after 1–2 h. The ratio of ricin to ricin chains in the translocation medium was found (using non-reducing gels) to vary with experiments (data not shown). We believe that these problems were because the dithiothreitol concentration we used in the translocation assay (1 mM) was not high enough to maintain the two ricin chains apart in the medium throughout the 3-h assay. It was not possible to increase this concentration in the translocation assay without reducing endosomal ricin (28).

This oxidation problem prevented us from investigating whether interchain reduction is essential for ricin translocation. Nevertheless, at least part of the endosomal ricin is reduced during translocation, since some separate chains are generated during the process (data not shown).

Ricin A Chain Retains Its Biological Activity after Translocation in the Cell-free Assay—Concerning the cytotoxic activity of ricin, resulting from the transfer of enzymatically active A chain in the cytosol, it was important to establish whether translocated ricin A was still able to arrest protein synthesis.

Inhibition of cell-free protein synthesis was observed using material released from either TF-RIA or ricin-loaded endosomes (Fig. 11) but not from TF-RIB endosomes (data not shown).

This inhibition was dependent on the translocation time in a kinetic pattern similar to the release of the A chain from endosomes (see Figs. 9 and 10), showing that translocated ricin A was enzymatically active.

**DISCUSSION**

Protein translocation across biological membranes is now becoming well documented. Reconstitution of the process after membrane solubilization has been achieved in both eucaryotic and prokaryotic systems (34, 35) allowing further characterization of all molecules implicated in the process. Ricin is a favorite toxin used in building immunotoxins, but despite its interest for therapeutics and especially for immunotoxin efficiency (1, 2, 36, 37) ricin translocation is poorly understood. This is mainly because of the lack of a direct translocation assay, like those that proved useful in describing DT translocation (6, 7).

There are still several questions to be addressed concerning ricin translocation. The first involves its localization in the endocytic pathway. It has been argued that ricin can translocate from a variety of endocytic elements including the endosome (9–11), the TGN (1, 2, 4, 5, 36) or a post-TGN compartment (12, 38), which may actually be the endoplasmic reticulum (39). No endocytosed ricin has ever been detected deeper than the TGN using conventional electron microscopy techniques (38, 39), and it is still believed that ricin translocation is more efficient from this compartment (1, 2, 4, 5, 40). Numerous data point to the involvement of the TGN in the cytotoxic action of ricin; it was proposed to be either the actual site of translocation (1, 2, 4, 5) or indirectly implicated through synthesis of a ricin receptor recognized by sugar binding sites of ricin (17, 18). This cell component would only be required for ricin but not for ricin A chain-based immunotoxins (15).

Recent results obtained using BFA, which prevents exit of proteins from the Golgi apparatus to the TGN while leaving the latter functional (13, 14), favor the receptor hypothesis: BFA-treated cells are protected against ricin but not against immunotoxins (15). Hence, translocation could potentially occur from all endocytic compartments where this receptor is found. This is not yet documented, and the actual site of ricin translocation in the cell is still an open question (1, 2, 4, 5, 8–12, 15–19, 38–43).

There is already a fairly large amount of data implicating the endosome as a dead-end of ricin A entry in the cytosol (9–11, 40–42). There has been strong evidence obtained using ricin-gold which can be easily followed within the cell; although only about three times less cytotoxic than ricin (28), it is not routed to the TGN (4) but like some active ricin A immunotoxins (40–42) follows the endosome to lysosome pathway (4, 26). Translocation probably does not occur from the lysosomes; accordingly, only intact ricin A is recovered in the cytosol after intoxication with these immunotoxins (43). It is also well known that the most efficient immunotoxins are those that are degraded least rapidly (10, 40–42). Hence, ricin A from either ricin-gold or ricin A immunotoxins is able to cross the endosome membrane. The A chain of native ricin can probably do the same; some electron microscopic evidence of ricin translocation from the endosomal compartment has been presented (11).

In this study the release of labeled ricin from within isolated endosomes was observed in two different assays and was both

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**Ricin Translocation across Endosome Membrane**

**FIG. 10.** Both ricin chains translocate from \(^{125}\)I-ricin-loaded endosomes. The medium containing the proteins translocated from \(^{125}\)I-ricin-loaded endosomes was reduced and then incubated with lactose-agarose to separate the ricin chains as described under "Experimental Procedures." B chain (○, lactose-bound); A chain (●, lactose unbound).

**FIG. 11.** Translocated ricin A is enzymatically active. The translocation assay of endosomes loaded with either ricin (●) or Tf-RIA (○) was stopped after the indicated period of time at 37 °C. The medium was then freed of endosomes by ultracentrifugation, and its inhibitory effect on cell-free protein synthesis was tested (after reduction of the interchain disulfide bridge in the case of ricin) using reticulocyte lysates, as described under "Experimental Procedures."
temperature- and ATP-dependent. A fluid phase label (horseradish peroxidase) and a membrane-bound tracer (transferrin) were not released under these conditions, and we therefore believe that our assay identifies ricin, which is actively translocated across the endosome membrane. This was confirmed using transferrin conjugates, as discussed later.

Ammonium chloride and nigericin were shown to neutralize endosomal pH without inhibiting (but rather increasing) ricin cytotoxicity (1, 2). It was therefore not surprising to find that they did not impair ricin translocation from purified endosomes. We found that the energy required for the process was provided by ATP hydrolysis through a not yet defined mechanism. The system that provides the energy for ricin to cross the endosome membrane differs from that enabling DT translocation (the endosome-cytosol pH gradient (6, 7)).

The temperature dependence was stronger for ricin than for DT translocation. The results showing that ricin translocation was negligible below 20 °C are of special interest since ricin toxicity is inhibited at these temperatures in intact cells (1, 2). Hence, it seems that low temperatures interfere not only with ricin transfer along the endocytic pathway (2) but also with ricin translocation across the membrane. The protective effect of temperatures below 20 °C against ricin intoxication results from a combination of these two different effects.

Our data indicate that the longer the interval following the onset of internalization, the more efficient the ricin translocation. Hence ricin translocation is much more active from late than from early endocytic compartments. In the same conditions, a much weaker dependence of the DT translocation rate upon the time of endocytosis was observed (7). Previous studies suggested that there are changes in composition of membrane proteins along the endocytic pathway (44, 45), and it is therefore possible that proteins required for ricin translocation like the receptor (15, 17, 18) are more concentrated in later compartments.

Both ricin chains were found able to insert into lipid membrane (46), but, even though some free A chain has been recovered in the cytosol after endocytosis of an A chain antibody immunotoxin (49), to our knowledge there is only one report indicating that both ricin chains can be found in the cytosol (11). It was therefore fairly surprising to find that the B chain was capable of translocation. Nevertheless, we believe that results obtained using transferrin conjugates to direct ricin chains in the endosome only and using TnI-horse-radish peroxidase as control clearly revealed the translocation ability of both ricin chains. Since they were recovered in a biologically active form, data obtained using this translocation cell-free assay probably describe what happens inside the cell.

We found that ricin was able to reach the cytosol from the plasma membrane, indicating that endocytosis is not strictly required for ricin cytotoxicity. Translocation at this level was negligible compared with the activity observed in endosomes, so we do not believe that ricin molecules transported through the plasma membrane are significantly involved in ricin cytotoxicity. Ricin probably translocates not only from endosomes but also from other endocytic compartments (1, 2, 38, 40). The general mechanism used to cross the membrane is probably similar, and ATP hydrolysis is quite likely needed for ricin translocation from other organelles.

Only the A chain of DT is transferred through the membrane during translocation, with the B chain remaining in the endosome (7). Characterization of ricin (this paper) and DT (7) translocation properties revealed that these toxins use different strategies to enter the cytosol.

We showed previously that endosomes incubated in conditions similar to those used for the translocation assay might undergo fusion (25). Some fusion events could hardly occur during ricin translocation since cytosol, which is needed for efficient endosome fusion (25, 47), was absent in the translocation assay. Moreover, the endosomal compartments involved in each activity are different since fusion is most effective between early endosomes (25, 47), whereas we found ricin translocation to take place preferentially in late endosomes.

Not all endosome preparations allow ricin translocation; endosomes obtained from the same cells while using a gold-based density shift procedure (44) were not found to release their 125I-ricin in cell-free assays (data not shown).

In conclusion, this study of ricin translocation across the endosomal membrane in a cell-free system sheds some light on immunotoxin action. First, the effect of ammonium chloride and other agents that enhance ricin-based immunotoxin cytotoxicity is not located at the translocation level but is probably related to their effect on routing of the conjugate in intact cells (1, 2). Second, the fact that transferrin conjugates were only found to translocate from the endosome indicates that the most critical criteria for building active immunotoxins is endocytosis efficiency of the target antigen. This agrees with studies on comparative immunotoxin activity as a function of their directing antibody (1, 41, 48).

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