Translocation of Ricin A-chain into Proteoliposomes Reconstituted from Golgi and Endoplasmic Reticulum*

Aykut Bilge§, Concepcion V. Warners, and Oliver W. Press††

From the Departments of ††Biological Structure and †§Medicine, University of Washington, Seattle, Washington 98195 and the ‡Fred Hutchinson Cancer Research Center, Seattle, Washington 98104

Translocation to the cytosol is an essential and rate-limiting step in the cytotoxicity of the potent plant toxin ricin. In an attempt to study the mechanism of ricin A-chain (RTA) translocation in a cell-free assay, we have partially purified Golgi and endoplasmic reticulum from Jurkat cells by discontinuous sucrose gradient fractionation. The membranes of the organelle fractions were solubilized by the addition of sodium cholate and reconstituted into proteoliposomes by dialyzing out the detergent. The resulting vesicles supported cell-free translocation of RTA (as assessed by an enzyme protection assay) at a rate which was linearly dependent on the concentration of the vesicle preparation. Ricin B-chain (RTB) neither translocated into the vesicles, nor increased the efficiency of RTA translocation. Liposomes prepared from purified phospholipids were not capable of supporting RTA translocation. Furthermore, protease treatment or concanavalin A adsorption of proteins from lysates prior to vesicle reconstitution resulted in abrogation of the translocation process, suggesting that the protein components of organelle membranes are required for RTA translocation. Reconstitution of translocation-competent proteoliposomes from detergent-solubilized membranes of endoplasmic reticulum- and Golgi-enriched fractions provides a convenient cell-free system to study the mechanism of RTA translocation.

Ricin is a potent plant toxin which is employed in the preparation of immunotoxins for the therapy of cancer and autoimmune and infectious diseases (1, 2). It is composed of two polypeptide chains, the 32-kDa ribosome-inactivating chain, RTA, and the 33-kDa cell binding chain, RTB, which are linked to each other with a disulfide bond. Following endocytosis of ricin or immunotoxin by the target cell, RTA is transported to a cell compartment where it translocates to the cytosol and exerts its ribosome-inactivating effect (3). RTA translocation is the rate-limiting step in the cytotoxicity of ricin and immunotoxins (4). However, the intracellular site(s) and the mechanism of RTA translocation are poorly understood. Electron microscopic studies have shown that ricin is transported as far as the trans-Golgi network in the endocytic pathway (5). Treatment of cells with brefeldin A, which disrupts the Golgi structure and blocks the connection between the endocytic pathway and the secretory pathway, results in the protection of cells from the toxic effects of ricin and other related plant and bacterial toxins such as modecin, abrin, velen, and Shiga toxin, but not of Diphtheria toxin, which is known to translocate to the cytosol from endosomes (6, 7). Furthermore, brefeldin A protects cells from cholera toxin-induced elevation of intracellular cAMP (8). These studies suggest that the retrograde transport of ricin and related toxins into proximal compartments of the secretory pathway such as the Golgi stacks and ER may be important for efficient cytotoxicity. Addition of an A retrieval sequence, KDEL, onto the C-terminal end of RTA significantly increased the cytotoxicity of ricin (9) and RTA (10) lending credence to the concept that RTA translocates to the cytosol most efficiently from the ER. A recent study demonstrated that ricin translocation starts in the endosomes, but that translocation efficiency increases as the toxin is transported deeper into the endocytic pathway (11).

A major obstacle for the study of ricin translocation in organelles deep in the internalization pathway, such as the Golgi stacks and ER, is death of cells before they accumulate sufficient concentrations of ricin to permit detection and experimentation. To circumvent this obstacle and to study the mechanism of translocation of RTA in these organelles, we developed a cell-free system in which we reconstituted translocation-competent proteoliposomes from detergent-solubilized membranes of Golgi- and ER-enriched fractions of Jurkat cells. Using this system, we studied the kinetics and the membrane requirements of RTA translocation and the effect of RTB on the translocation of RTA.

EXPERIMENTAL PROCEDURES

Reagents—RTA and RTB were purchased from Inland Laboratories (Austin, TX); Triton X-100, proteinase K, papain, and concanavalin A-Sepharose beads were from Sigma; ultrapure sodium cholate and Nonidet P-40 were from Calbiochem; immobilized protease Sg (a nonspecific protease) and immobilized papain were from Pierce; and phosphatidylincholine, phosphatidylethanolamine, and phosphatidylinositol were from Avanti Polar Lipids Inc. (Alabaster, AL). A murine IgG₁ antibody recognizing the luminal domain of calnexin (AF8) (12) was a generous gift of Dr. Michael Brenner. A murine IgG₁ antibody targeting the luminal domain of the MPR (86f7) was kindly provided by Dr. Stuart Kornfeld. Murine IgG₁ was obtained by purification of monoclonal antibody DA 4.4 from ascitic fluid by affinity chromatography on Sepharose-staphylococcal protein A (Sigma). j urkat cells were purchased from ATCC (Rockville, MD) and maintained as described previously (13).

Radioiodination—RTA, RTB, mouse IgG₁, anti-MPR antibody, and anti-calnexin antibody were radioiodinated as described previously (13). The specific activities of ¹²⁵I-RTA and ¹³¹I-RTB were 14 and 70 Ci/mmol⁻¹, respectively.

Discontinuous Sucrose Gradient Fractionation—Fractionation methods were adapted from previous studies (14, 15). All procedures were carried out on ice. 1 × 10⁹ j urkat cells were suspended in buffer A (50 mM TEA, 50 mM KCl, 2 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 0.5 mM cAMP) and carried out on ice. 1 × 10⁹ j urkat cells were suspended in buffer A (50 mM TEA, 50 mM KCl, 2 mM Mg(OAc)₂, 1 mM EDTA, 1 mM DTT, 0.5 mM cAMP) and
phenylmethylsulfonyl fluoride) containing 0.25 M sucrose. The cells were homogenized with 30 strokes of a Dounce homogenizer (Kontes Inc., Moline, IL), and the nuclei and intact cells were removed by centrifuging at 10,000 × g for 5 min at 4 °C. The sucrose concentration of the supernatant was adjusted to 1.3 M by the addition of an appropriate amount of 2.5 M sucrose in buffer A and 4.5 ml of the resultant mixture placed in each of two Beckman ultracentrifuge tubes. The 1.3 M sucrose gradient mixture was overlaid with 5 ml of 1.2 M sucrose in buffer A and with 3 ml of 0.8 M sucrose in buffer A. After ultracentrifugation for 2.5 h at 4 °C in a SW40 rotor at 94,000 × g, 1-ml fractions were collected and numbered from bottom to top. Fractions were then analyzed by enzyme assays and by electron microscopy as described previously (16).

Enzyme and Protein Assays—The Golgi-specific enzyme, galactosyltransferase, was assayed by measuring the transfer of UDP-[U-14C]galactose (Amersham Corp., Arlington Heights, IL) to ovalbumin (Sigma) as described previously (17). The lysosomal enzyme, β-galactosidase, was assayed using a colorimetric method (18). The ER-associated enzyme, glucose-6-phosphatase, was assayed by measuring the release of inorganic phosphate from glucose 6-phosphate (Sigma) (17, 19). Protein concentrations of the fractions were determined by biocinchonic acid (BCA) assay (Pierce).

Reconstitution of Proteoliposomes—The reconstitution method was adapted from the procedure described by Nichitta and Blobel (20, 21). Fractions 12 and 10 (Golgi) of the gradient were diluted to 0.5 M sucrose concentration by the addition of the appropriate amount of buffer B (0.25 M sucrose, 50 mM TEA, 1 mM DTT), and 3-ml aliquots were placed in tubes on ice. The pellet (fraction 1) was suspended in 1.1 ml of buffer B and supplemented by an equal amount of buffer D (0.25 M sucrose, 50 mM TEA, 1 mM DTT) to strip ribosomes. Following 15-min incubation on ice, this sample was placed in tubes underlaid with 0.8 M of buffer E (0.25 M sucrose, 50 mM TEA, 1 mM DTT) to strip cytosol. Following 15-min incubation on ice, this sample was placed in tubes underlaid with 0.8 M of buffer F (0.4 M sucrose, 0.4 M KCl, 20 mM Tris, pH 7.6, 1.5 mM MgCl2, 1 mM EDTA). The membranes were solubilized by the addition of sodium cholate and reconstituted into vesicles by dialysis as described previously (20, 21). Vesicle concentrations were standardized prior to the performance of assays so that 250 μl of each preparation yielded an absorbance of 0.2 at 405 nm using a EL-310 model enzyme-linked immunosorbent assay reader (Bio-Tek Instruments, Inc., Winooski, VT).

To study the translocation of preprolactin, two-dimensional gel electrophoresis was performed. The reconstituted proteoliposomes were topologically reconstituted vesicles produced are topologically reconstituted proteoliposomes should permit “retrograde” translocation or in orientations opposite their native configurations (21) to study translocation of preprolactin. Using this method-

Translocation of Ricin A-chain

J urkat cell homogenates were fractionated by centrifugation on a discontinuous sucrose gradient composed of 1.3, 1.2, and 0.8 M layers. Twelve fractions were collected (numbered from the bottom) and examined for their organelle content by electron microscopy and enzymatic analysis (Fig. 1). Relatively pure ER was recovered in fraction 1 as assessed by the presence of rough microsomes by electron microscopy (data not shown) and by the recovery of glucose-6-phosphatase activity. The microsome-bound ribosomes in this fraction were removed by washing with EDTA. Fraction 5 (1.3 M/1.2 M interface) was composed of lysosomes, mitochondria, and smooth ER by electron microscopic criteria (data not shown). Most of the lysosomal β-galactosidase activity was contained in this fraction. Galactosyltransferase activity was recovered in fractions 10 and 11, indicating the presence of Golgi in these fractions. Fraction 10 (1.2 M/0.8 M interface) also contained some contaminating endosomes and surface membranes (data not shown). At this point, the cellular structures were abundant in fraction 12 by electron microscopy (data not shown), this fraction contained very little β-galactosidase, glucose-6-phosphatase, or galacto-
syltransferase activity. A protein profile of the fractions is presented in Fig. 1.

FIG. 1. Characterization of fractions obtained by discontinuous sucrose gradient centrifugation. J urkat cell homogenates were disrupted with a Dounce homogenizer and fractionated on discontinuous sucrose gradients by ultracentrifugation. Harvested fractions were analyzed for their activities for galactosyltransferase (○), a Golgi-specific enzyme; β-galactosidase (□), a lysosome-specific enzyme; and glucose-6-phosphatase (●), an ER-associated enzyme (upper panel), as well as for protein concentrations (▲) (bottom panel).

RESULTS

Purification and Characterization of Golgi and ER

The proteoliposome reconstitution system we have employed is identical to the system employed by Nichitta and Blobel (20, 21) to study translocation of preprolactin. Using this methodology, the reconstituted vesicles produced are topologically restructured so that vectorially oriented membrane proteins are redistributed variably either in their native membrane orientations or in orientations opposite their native configurations (i.e. some luminal domains become abluminal). Such “reconstituted” proteoliposomes should permit “retrograde” translocation from the exterior to the interior of the vesicles. To confirm that the expected topological reorganization of the proteolipo-
some membranes had occurred, we tested the ability of 125I-labeled monoclonal antibodies specific for the luminal domains of integral Golgi and ER membrane proteins, the mannose 6-phosphate receptor and calnexin, respectively, to bind to the

Fraction

Relative Concn (%)
Translocation of Ricin A-chain

Fig. 2. Topology of the reconstituted vesicles. Vesicles reconstituted from Golgi- (●) and ER-enriched (○) fractions were incubated with 125I-IgG1, 125I-anti-MPR, and 125I-anti-calnexin for 45 min at room temperature and then diluted with buffer G and pelleted. The vesicle-associated antibody was determined by γ counting the radioactivity of the pellet and expressed as the percentage of the total. The total counts/min (bound + unbound) was 4,141 for 125I-IgG1, 22,732 for 125I-anti-MPR, and 9,782 for 125I-anti-calnexin. One of two concordant experiments is shown.

exterior of the reconstituted vesicles. As shown in Fig. 2, specific binding of 125I-anti-MPR antibody confirmed the presence of the luminal domain of the mannose 6-phosphate receptor on the exterior surface of the vesicles reconstituted from Golgi-enriched fractions. Similarly, luminal determinants of calnexin were detected on the exterior surface of the vesicles reconstituted from ER-enriched fractions using 125I-anti-calnexin antibody (Fig. 2). A nonspecific mouse IgG1 used as a control reagent bound minimally to the vesicles (Fig. 2).

Translocation of RTA into Vesicles Reconstituted from Golgi- and ER-enriched Fractions

RTA Preincubated with Vesicles Reconstituted from Golgi- and ER-enriched Fractions Is Protected from Subsequent Papain Digestion—Vesicles were reconstituted identically from fractions 1 (ER-enriched), 10 (Golgi-enriched), and 12 and were standardized to the same concentration spectrophotometrically before utilizing in translocation assays. 125I-RTA was incubated with and without vesicles reconstituted from fractions 1 (ER), 10 (Golgi), and 12 for 1 h at 37 °C. Samples were then subjected to papain digestion on ice, and the degradation of 125I-RTA was assessed by SDS-PAGE and autoradiography. As shown in Fig. 3a, 125I-RTA was completely digested in the samples preincubated without vesicles or with vesicles reconstituted from fraction 12; however, a quantity of 125I-RTA was protected from digestion in samples preincubated with vesicles reconstituted from Golgi- and ER-enriched fractions. Similar results were obtained with proteinase K digestion (data not shown).

Protection from Papain Digestion Is Abrogated by Detergent Disruption of the Vesicles—125I-RTA was incubated with and without vesicles reconstituted from Golgi- and ER-enriched fractions in the presence and absence of 1% Nonidet P-40 for 1 h at 37 °C. The samples were then subjected to papain digestion and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 3b, the integrity of the vesicles was required for protection from papain digestion, since 125I-RTA was completely digested when the vesicles were disrupted by detergents. Similar results were obtained when Nonidet P-40 was added after the incubation of the vesicles with RTA (data not shown). These data suggest that a quantity of 125I-RTA is protected from papain digestion by translocating into the vesicles reconstituted from Golgi- and ER-enriched fractions and that the vesicles reconstituted from fraction 12 are not competent for RTA translocation.

Linear Relationship between the Amount of Translocated RTA and the Concentration of the Vesicle Preparation

125I-RTA was incubated with and without vesicles at the dilutions indicated in Fig. 4 in the presence and absence of 1% Triton X-100 for 1 h at 37 °C. The samples were then subjected to papain digestion and analyzed by SDS-PAGE and autoradiography. As shown in Fig. 4, the amount of 125I-RTA that translocated was dependent on the concentration of the vesicle preparation. Dilution of the vesicles resulted in lesser protection from papain digestion (upper panel). Densitometric analysis of the lanes revealed that there was a linear relationship between the amount of translocated RTA and the concentration of the vesicles (lower panel).

Effect of Incubation Time on the Translocation of RTA into Reconstituted Vesicles

125I-RTA was incubated with and without vesicles for 0.5, 1, 2, and 4 h time points at 37 °C. The samples were then subjected to papain digestion and analyzed by SDS-PAGE and autoradiography (Fig. 5, upper panel). The lanes from the autoradiographs of two experiments were analyzed by densitom-
also abrogated the translocation competence of the subse-

priortovesiclereconstitution. Thismethodofproteindepletion

trough ConA columns to deplete glycoprotein constituents

extracts of Golgi- and ER-enriched fractions were passed

conclusions derived from the protease experiments. Cholate

treated cholate extracts (upper panel) proteases (data not shown).

reconstituted vesicles as illustrated in Fig. 6 (lower panel).

RTB Does Not Translocate into Reconstituted Vesicles

RTB, the cell binding domain of ricin, is a potentiator of RTA

immunotoxins which is postulated to facilitate RTA transloca-

tion (23–27). We tested the ability of RTB to translocate into

reconstituted vesicles. 125I-RTB was incubated with and with-

out vesicles reconstituted from Golgi-enriched fractions in the

presence and absence of 1% Nonidet P-40 for 1 h at 37 °C. The

samples were then subjected to papain digestion and analyzed

by SDS-PAGE and autoradiography. RTB did not translocate

into the reconstituted vesicles, but was completely digested by

papain (Fig. 7). A similar result was obtained from an experi-

ment in which vesicles reconstituted from the ER-enriched

fraction were used (data not shown).

RTB Protects RTA from Enzyme Digestion

We tested the effect of RTB on the translocation of RTA into

reconstituted vesicles. 125I-RTA was incubated with and with-

out vesicles reconstituted from the ER-enriched fraction in the

presence and absence of unlabeled RTB for 1 h at 37 °C. The

samples were then subjected to proteinase K digestion and analyzed

by SDS-PAGE, autoradiography, and densitometry. The results

are shown in Fig. 8. In the presence of vesicles, 125I-RTA was

protected from digestion by proteinase K (lane C). Addition

of RTB in the absence of vesicles also resulted in the protec-

tion of 125I-RTA from proteolysis (lane F). The presence

of RTB together with vesicles resulted in a protection that was

not significantly greater than the sum of the protection ob-

tained in the presence of either RTB or vesicles alone (lane G)

as assessed by densitometric analysis of the lanes in six sep-

arate experiments (data not shown). Nonidet P-40 abrogated

the protective effect of vesicles but not of RTB on the digestion

of RTA (lanes D and H). These data suggest that RTB is not

essential for effective translocation of RTA.

DISCUSSION

Several independent lines of investigation have suggested

that most plant and bacterial toxins, including ricin, translo-

cate to the cytosol most efficiently from organelles deep in the

internalization pathway (e.g. the trans-Golgi region or endo-

plasmic reticulum). Ricin has been demonstrated in the trans-

•

tery and plotted as shown in the lower panel of Fig. 5. The

results indicate that the amount of RTA that translocates

increases with the incubation time, reaching a plateau of approxi-

mately 25% of total RTA in 1 h.

Liposomes Prepared from Purified Phospholipids

Do Not Support RTA Translocation

Liposomes were prepared from purified phosphatidylcholine,

phosphatidylethanolamine, and phosphatidylserine and then

tested for RTA translocation competence. 125I-RTA was

incubated with and without vesicles at the indicated dilutions for 1

h at 37 °C. The samples were then subjected to papain diges-

tion and analyzed by SDS-PAGE and autoradiography. 125I-

RTA was completely digested even in the presence of vesicles,

suggesting that the protein component of the Golgi or ER

fractions is required for the translocation of RTA (data not

shown).

Removal of the Protein Components of Golgi and ER

Vesicles Abrogates RTA Translocation

To further investigate the role of the protein constituents of

Golgi and ER proteoliposomes in the translocation process, we

reconstituted cholate extracts with immobilized papain and protease

Sg (a nonspecific protease) for 2, 18 and 42 h prior to vesicle

reconstitution. After removal of the immobilized proteases, ves-

icles were reconstituted and tested for their competence for

RTA translocation. As shown in Fig. 6 (upper three panels),

vesicles reconstituted from Golgi-enriched fractions after 2 h of

protease treatment retained translocation competence, whereas

longer protease treatments completely abrogated the ability of

reconstituted vesicles to support translocation. Similar

conclusions were reached using vesicles reconstituted from

ER-enriched fractions, although translocation was only re-

duced by 47% after 42 h of incubation with immobilized pro-

teases (data not shown).

An alternative protein depletion approach corroborated

the conclusions derived from the protease experiments. Cholate

extracts of Golgi- and ER-enriched fractions were passed

through ConA columns to deplete glycoprotein constituents

prior to vesicle reconstitution. This method of protein depletion

also abrogated the translocation competence of the subse-

The amount of the translocated RTA at each time point was determined

densitometrically from the lanes and plotted (lower panel). (○, experiment 1; ●, experiment 2).

The results are shown in Fig. 8. In the presence of vesicles, 125I-RTA was protected from digestion by proteinase K (lane C).

Addition of RTB in the absence of vesicles also resulted in the protection of 125I-RTA from proteolysis (lane F). The presence of RTB together with vesicles resulted in a protection that was not significantly greater than the sum of the protection obtained in the presence of either RTB or vesicles alone (lane G) as assessed by densitometric analysis of the lanes in six separate experiments (data not shown). Nonidet P-40 abrogated the protective effect of vesicles but not of RTB on the digestion of RTA (lanes D and H). These data suggest that RTB is not essential for effective translocation of RTA.

Fig. 5. Effect of incubation time on the translocation of RTA.

125I-RTA was incubated with and without vesicles for the indicated

times and then digested with 1 unit/ml papain for 75 min on ice.

Degradation was analyzed by SDS-PAGE and autoradiography. The

autodigraphy of two separate experiments are shown (upper panel).

The amount of the translocated RTA at each time point was determined

densitometrically from the lanes and plotted (lower panel). (○, experiment 1; ●, experiment 2).

Effect of protein depletion on translocation of RTA.

125I-RTA was incubated with vesicles reconstituted from protease-
treated cholate extracts (upper three panels) or ConA-adsorbed cholate

extracts (lower panel) of the Golgi-enriched fraction, digested with protease

K, and analyzed by SDS-PAGE and autoradiography. Sim-

ilar results were obtained in experiments using ER-enriched fractions

for vesicle reconstitution (data not shown).
**Inability of RTB to translocate into reconstituted vesicles.** RTB was incubated with and without vesicles reconstituted from Golgi-enriched fractions in the presence and absence of 1% Nonidet P-40 and then digested with 1 unit/ml papain for 75 min. Degradation of 125I-RTB was analyzed by SDS-PAGE, autoradiography, and densitometry. Similar results were obtained using proteoliposomes reconstituted from ER-enriched membranes (data not shown).

**Effect of RTB on the translocation of RTA into reconstituted vesicles.** RTB was incubated with and without vesicles reconstituted from ER-enriched fractions in the presence and absence of 0.1 μg of unlabeled RTB and then digested with 1 unit/ml proteinase K. Degradation of 125I-RTA was analyzed by SDS-PAGE, autoradiography, and densitometry. Similar results were obtained using proteoliposomes reconstituted from Golgi-enriched membranes (data not shown).

Golgi network by electron microscopic studies (5, 6). Furthermore, brefeldin A treatment has protected the cells from the detrimental effects of most related bacterial and plant toxins, suggesting that the organelles of the secretory pathway (Golgi and ER) might be involved in the action of these toxins (6–8). The increased cytotoxicity of the KDEL-modified RTA has provided additional evidence for the trafficking of RTA to Golgi and ER for efficient translocation (9, 10). Unfortunately, it has been very difficult to study toxin translocation from the Golgi or ER, because the extreme potency of these reagents results in cell death before appreciable concentrations of toxin accumulate in these compartments. For example, it has been estimated that one ricin molecule can inactivate 1500 ribosomes per minute and that this rate is sufficient to kill a cell (28, 29).

Although the efficiency of the translocation process is not known with certainty, it appears plausible to assume that most standard assays are insufficiently sensitive to detect toxin concentrations capable of causing irreversible cytotoxicity. Consequently, we have developed an in vitro translocation assay based on proteoliposomes reconstituted from purified Golgi- or ER-enriched membranes by adapting methodology which has proven indispensable for the study of translocation of secretory proteins from ribosomes to the ER lumen (20, 21, 30). As demonstrated in the present study, translocation-competent proteoliposomal vesicles from detergent solubilized membranes of Golgi- and ER-enriched fractions provide convenient cell-free systems for the study of RTA translocation.

Our experiments show that RTA is able to translocate efficiently into reconstituted vesicles derived from either Golgi- or ER-enriched fractions of Jurkat cells in a manner linearly dependent on the vesicle concentration. The kinetics of the translocation process show that the amount of RTA that translocates increases linearly for 1 h and then plateaus. It appears likely that the cessation of translocation after 1 h in this reconstituted system is due to depletion of an endogenous (organelle membrane-derived) energy source that drives the translocation machinery. In their recent study of ricin translocation across endosomal membranes, Beaumelle et al. (11) showed that ricin translocation in endosomes is ATP-dependent and stops after 30 min unless exogenous ATP is added. The difference in the duration of translocation without exogenous ATP between the present study and that of Beaumelle et al. (11) may reflect inherent differences in the distribution of energy-generating systems between Golgi, ER and endosomes. RTB is known to augment the toxicity of RTA (23–27), although the mechanism underlying this potentiating effect is poorly understood. It has long been postulated that RTB facilitates translocation of RTA in a manner analogous to that proposed for diphtheria toxin B-chain which is believed to insert into endosomal membranes at acidic pH values, forming a channel for A-chain translocation to the cytosol (23, 24, 26, 31). Alternatively, Lord and co-workers (32, 33) have suggested that RTB may subserve an intracellular shuttling function by binding to intracellular galactose residues expressed by molecules along the internalization pathway, thereby “ratcheting” RTA along the endocytic pathway to its translocation site. Finally, recent studies have shown that RTB protects RTA from degradation by endosomal and lysosomal proteolytic enzymes and may thereby enhance the cytotoxicity of RTA (13).

In the present study, we tested the ability of RTB to translocate into reconstituted Golgi and ER vesicles and its effect on the translocation of RTA. RTB neither translocated by itself, nor augmented the amount of RTA that translocated into reconstituted vesicles. In contrast to our findings with reconstituted Golgi and ER proteoliposomes, Beaumelle et al. (11) found that RTB was able to translocate across the membranes of endosomes purified by gradient centrifugation. The discrepancy between our findings and those of Beaumelle et al. (11) may reflect methodologic differences or could result from structural differences in the organelles studied. The membrane component which facilitates translocation of RTB in endosomes may not exist in Golgi and ER, or alternatively, it could be lost or inactivated in the reconstitution process.

One of the most active areas of current research in cell biology concerns investigations into the mechanisms involved in protein transport across biological membranes. Studies of peptide (34, 35) and nascent chain (36) translocation across membranes of the endoplasmic reticulum, mitochondria (37), and bacterial surface membranes (38) have demonstrated that protein channels exist which facilitate transmembrane protein transport (e.g. “translocon” protein channels for nascent secretory proteins (36, 39) and the “TAP transporter” channels (34, 35) for targeting cytosolic peptides to class I major histocompatibility complex molecules). Since our present studies indicate that liposomes prepared from purified phospholipids or from cellular fractions depleted in membrane glycoproteins are unable to support the translocation of RTA, it appears likely that proteinaceous constituents of Golgi and ER membranes play an integral role in the translocation of ricin A-chain and other toxins. As suggested by Wales et al. (10), it is conceivable that RTA utilizes previously unidentified translocation components of the ER (i.e. the translocon complex) in a retrograde direction, from lumen to the cytosol. Alternatively, heretofore unrecognized protein channels may be employed for toxin translocation.

Recent studies by Theuer et al. (40, 41) employing an in vitro translation/translocation system suggest that a truncated form of Pseudomonas exotoxin (PE37) can insert into the membrane of canine pancreatic microsomes if guided by the propeptide signal sequence, but that a “stop-transfer” sequence in domain II of the toxin arrests translocation and releases the toxin from the microsomes before it can transfer into the lumen of the microsomes. Our data suggest that a less complicated interaction occurs between ricin A-chain and Golgi/ER proteoliposomes and that full translocation of the molecule occurs in the absence of a signal sequence and without interruption by a stop transfer sequence. We believe that translocation-compe-
tent proteoliposomes such as those described in this report will prove to be as useful for the molecular dissection of the structural components involved in the translocation of plant and bacterial toxins as they have been in studying the translocation of secretory proteins (20, 21, 30).

REFERENCES
1. Press, O. W. (1991) Biotherapy 3, 65–76
2. Vitetta, E. S., and Thorpe, P. E. (1991) Semin. Cell Biol 2, 47–58
3. van Deurs, B., Sandvig, K., Petersen, O. W., and Olsnes, S. (1990) Trafficking of Bacterial Toxins (Saelinger, C. B., ed) pp. 91–119, CRC Press, Inc., Boca Raton, Fl.
4. Hudson, T. H., and Neville, D. M., Jr. (1987) J. Biol. Chem. 262, 16484–16494
5. van Deurs, B., Sandvig, K., Petersen, O. W., Olsnes, S., Simons, K., and Griffiths, G. (1988) J. Cell Biol. 106, 253–267
6. Sandvig, K., Prydz, K., Hansen, S. H., and van Deurs, B. (1991) J. Cell Biol. 115, 971–981
7. Hudson, T. H., and Grilli, F. G. (1991) J. Biol. Chem. 266, 18586–18592
8. Nambiar, M. P., Oda, T., Chen, C., Kuwazuru, Y., and Wu, H. C. (1993) J. Biol. Chem. 268, 23661–23669
9. Laemmli, U. K. (1970) Nature 227, 680–685
10. Nunnari, J., and Walter, P. (1992) Curr. Opin. Cell Biol. 4, 573–580
11. London, E. (1992) Biochim. Biophys. Acta 1135, 25–51
12. Newton, D. L., Wales, R., Richardson, P. T., Walbridge, S., Saxena, S. K., Ackerman, E. J., Roberts, L. M., Lord, J. M., and Youle, R. J. (1992) J. Biol. Chem. 267, 11917–11922
13. Wang, L., and Berthet, J. (1994) J. Cell Biol. 125, 125–135
14. Walsh, C., and Berthet, J. (1994) J. Biol. Chem. 269, 3221–3227
15. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 44–93
16. Geissler, F., Anderson, S. K., Venkatesan, P., and Press, O. (1992) Cancer Res. 52, 2907–2915
17. Beaufay, H., Amar-Costescu, A., Feytmans, E., Thines-Sempoux, D., Wibo, M., Robbi, M., and Berthet, J. (1974) J. Cell Biol. 61, 188–200
18. Rome, L. H., Garvin, A. J., Aliotta, M. M., and Neufeld, E. E. (1979) Cell 17, 143–153
19. Ames, B. N. (1966) Methods Enzymol. 14, 15–14
20. Nicchitta, C. V., and Blobel, G. (1990) Cell 60, 259–269
21. Nicchitta, C., Migliaccio, G., and Blobel, G. (1991) Methods Cell Biol. 34, 263–285
22. Laemmli, U. K. (1970) Nature 227, 680–685
23. McIntosh, D. P., Edwards, D. C., Cumber, A. J., Parnell, G. D., Dean, C. J., Ross, W. C. J., and Forrester, J. A. (1983) FEBS Lett. 164, 17–20
24. Youle, R. J., and Neville, D. M., Jr. (1982) J. Biol. Chem. 257, 1598–1601
25. Vitetta, E. S., Cushley, W., and Uhr, J. W. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 6332–6335
26. Vitetta, E. S. (1986) J. Immunol. 136, 1880–1887
27. Warronczak, E. J., Drake, A. F., Watson, G. J., Thorpe, P. E., and Vitetta, E. S. (1988) Biochim. Biophys. Acta 971, 55–62
28. Carrasco, L., Fernandez-Puentes, C., and Vazquez, D. (1975) Eur. J. Biochem. 54, 499–503
29. Eiklid, K., Olsnes, S., and Pihl, A. (1980) Exp. Cell Res. 126, 321–326
30. Goričk, D., Hartmann, E., Prehn, S., and Rapoport, T. A. (1992) Nature 357, 47–52
31. London, E. (1992) Biochim. Biophys. Acta 1132, 25–51
32. Newton, D. L., Wales, R., Richardson, P. T., Walbridge, S., Saxena, S. K., Ackerman, E. J., Roberts, L. M., Lord, J. M., and Youle, R. J. (1992) J. Biol. Chem. 267, 11917–11922
33. Lord, J. M., Wales, R., Pitcher, C., and Roberts, L. M. (1992) Biochem. Soc. Trans. 20, 734–738
34. Monaco, J. J. (1992) J. Neurosci. 12, 173–179
35. Androlewicz, M. J., and Cresswell, P. (1994) Immunity 1, 7–14
36. Nunnari, J., and Walter, P. (1992) Curr. Opin. Cell Biol. 4, 573–580
37. Wennhues, U., and Neupert, W. (1992) Bioessays 14, 17–23
38. Swidersky, U. E., Hoffshulte, H. K., and Müller, M. (1990) EMBO J. 9, 1777–1785
39. Gilmore, R. (1993) Cell 75, 589–592
40. Theuer, C., Kasturi, S., and Pastan, I. (1994) Biochemistry 33, 5894–5900
41. Theuer, C. P., Buchner, J., Fitzgerald, D., and Pastan, I. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7774–7778
42. Theuer, C., Kasturi, S., and Pastan, I. (1994) Biochemistry 33, 5894–5900
43. Theuer, C. P., Buchner, J., Fitzgerald, D., and Pastan, I. (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 7774–7778