Cholera Toxin Is Exported from Microsomes by the Sec61p Complex

Anton Schmitz, Helga Herrgen, Alexandra Winkeler, and Volker Herzog

Institut für Zellbiologie, Rheinische Friedrich-Wilhelms-Universität Bonn, 53121 Bonn, Germany

Abstract. After endocytosis cholera toxin is transported to the endoplasmic reticulum (ER), from where its A1 subunit (CTA1) is assumed to be transferred to the cytosol by an as-yet unknown mechanism. Here, export of CTA1 from the ER to the cytosol was investigated in a cell-free assay using either microsomes loaded with CTA1 by in vitro translation or reconstituted microsomes containing CTA1 purified from *V. cholerae*. Export of CTA1 from the microsomes was time- and adenosine triphosphate–dependent and required lumenal ER proteins. By coimmunoprecipitation CTA1 was shown to be associated during export with the Sec61p complex, which mediates import of proteins into the ER. Export of CTA1 was inhibited when the Sec61p complexes were blocked by nascent polypeptides arrested during import, demonstrating that the export of CTA1 depended on translocation-competent Sec61p complexes. Export of CTA1 from the reconstituted microsomes indicated the de novo insertion of the toxin into the Sec61p complex from the lumenal side. Our results suggest that Sec61p complex–mediated protein export from the ER is not restricted to ER-associated protein degradation but is also used by bacterial toxins, enabling their entry into the cytosol of the target cell.

Key words: cholera toxin • endoplasmic reticulum • Sec61p complex • translocation • endoplasmic reticulum–associated protein degradation

Introduction

Several bacterial and plant toxins covalently modify cytosolic proteins of mammalian cells. To reach their target proteins these toxins have to gain access to the cytosol of the intoxicated cell. One group of these toxins, exemplified by diphtheria toxin, undergoes a conformational change induced by the acidic pH inside endosomes or lysosomes, allowing these toxins to penetrate directly through the lipid phase of the endosomal–lysosomal membrane (for review see Olsnes et al., 1988). In contrast to this group of toxins, the intoxication by cholera toxin, *E. coli* heat labile enterotoxin, *Pseudomonas aeruginosa* exotoxin A, Shiga toxin, ricin, and modeccin can be prevented by preincubating the cells with brefeldin A (Yoshida et al., 1991; Donta et al., 1993; Nambiar et al., 1993; Orlandi et al., 1993). Brefeldin A inhibits transport between the ER and the Golgi apparatus. Therefore, this second group of toxins is assumed to be transported from the plasma membrane via the Golgi apparatus to the ER and to cross the membrane there (for reviews see Pelham et al., 1992; Hazes and Read, 1997; Lord and Roberts, 1998; Lencer et al., 1999). Indeed, the appearance in the ER of Shiga toxin (Sandvig et al., 1992; Johannes et al., 1997) and of cholera toxin A1 subunit (CTA1)[1] (Majoul et al., 1996) has been demonstrated. For a mutant ricin containing additional *N*-glycosylation sites at its COOH terminus, it was shown that it reached the cytosol after being glycosylated in the ER (Rapak et al., 1997). However, direct experimental evidence is lacking that would indicate whether the transfer to the cytosol takes place from the ER. It is also unknown whether this transfer is accomplished by a change in the conformation of the toxins and direct penetration through the lipid phase of the membrane, or whether the translocation apparatus of the ER is used by the toxins to achieve their transfer across the membrane.

The latter speculation is attractive in light of recent findings on the degradation of misfolded secretory and membrane proteins, a process called ER-associated protein degradation (for review see Brodsky and McCracken, 1999; Plemper and Wolf, 1999). Degradation by cytosolically localized proteasomes was first demonstrated for the misfolded membrane protein cystic fibrosis transmembrane conductance regulator (Jensen et al., 1995; Ward et al., 1995), and for the misfolded secretory protein α1-antitrypsin (α1-AT) Z in mammalian cells (Qu et al., 1996), as

---

Address correspondence to Dr. Anton Schmitz, Institut für Zellbiologie, Rheinische Friedrich-Wilhelms-Universität Bonn, Ulrich-Haberland-Strasse 61a, 53121 Bonn, Germany. Tel.: 49-228-735306. Fax: 49-228-735302. E-mail: anton-schmitz@uni-bonn.de

[1]*Abbreviations used in this paper:* α1-AT, α1-antitrypsin; BiP, heavy chain binding protein; CPY*, mutant form of carboxypeptidase Y; CTA1, cholera toxin A1 subunit; DHFR, dihydrofolate reductase; RNC, ribosome nascent chain complex; TX, Triton X.

well as for mutant forms of carboxypeptidase Y (CPY*) (Hiller et al., 1996) and for unglycosylated pro–α-factor (Werner et al., 1996) in yeast. Degradation of these proteins by proteasomes implied their transfer from the ER to the cytosol. Recently, it was shown that degradation intermediates of the major histocompatibility complex class I heavy chain (Wiertz et al., 1996) and of cystic fibrosis transmembrane conductance regulator (Bebök et al., 1998) were associated with the Sec61p complex, suggesting that the Sec61p complex might be involved in the transfer of degradation substrates from the ER to proteasomes. The function of the Sec61p complex in delivering misfolded pro–α-factor (Pilon et al., 1997) and CPY* (Plemper et al., 1997) to proteasomes was implicated by genetic studies in yeast. It is as yet unknown whether the misfolded proteins had completely left the Sec61p complex before their export was initiated and therefore required the de novo insertion into the channel from the lumenal side. However, de novo insertion is the prerequisite for the Sec61p complex–mediated export of proteins such as bacterial toxins that did not enter the ER via the Sec61p complex.

In contrast to its possible function in protein export, the function of the Sec61p complex in the import of proteins into the ER is well established (for reviews see Rapoport et al., 1996; Johnson, 1997). Proteins enter the ER through a channel that provides an aqueous environment for the translocating polypeptide chain (Crowley et al., 1994). The Sec61p complex was shown by EM to form a tunnel through the lipid bilayer (Hanein et al., 1996), which is in alignment with the channel formed by the large ribosomal subunit (Beckmann et al., 1997), suggesting that the Sec61p complex is the protein-conducting channel. Indeed, translocation was reconstituted in proteoliposomes containing only the Sec61p complex, the signal recognition particle receptor, and the translocating chain-associating membrane protein (Görlich and Rapoport, 1993). Under certain conditions the Sec61p complex alone was sufficient for translocation of proteins across the ER membrane (Jungnickel and Rapoport, 1995).

Based on the studies on the ER-associated protein degradation, protein translocation by the Sec61p complex seems to be possible in both directions, i.e., into and out of the ER. Therefore, we asked the question whether CTA1 utilizes the Sec61p complex to cross the membrane of the ER and enter the cytosol of the target cell. For this purpose we reconstituted the transfer of CTA1 from the lumen of the ER to the cytosol in a cell-free system using ER-derived, translocation-competent microsomes. We show that the export of CTA1 from the microsomes is indeed mediated by the Sec61p complex.

Materials and Methods

Plasmids and In Vitro Transcription

For the construction of plasmid pSPCTA1 coding for the complete CTA1 (amino acids 1–212 of cholera toxin A subunit; sequence data available from EMBL/GenBank/DDBJ/Swissprot under accession no. P01555) (Mekalanos et al., 1983) the region of pRIT10841 (no. 39053; ATCC) corresponding to nucleotides 501–1151 of the cholera toxin gene (sequence data available from EMBL/GenBank/DDBJ under accession no. X00171) (Mekalanos et al., 1983) was amplified by PCR using a 3′-primer, inserting

a stop codon after amino acid 212, and subcloned into the plasmid vector pSVSport1 (Life Technologies). For the construction of CTA1Ins, the cDNA coding for B5Ser-proinsulin (Schmitz et al., 1995), except for the signal peptide and the first two amino acids of the mature protein, was fused in frame to the last codon of CTA1. The constructs were controlled by DNA sequencing (SequiServe). For the generation of CTA1Δ137 (encoding the first 75 amino acids of CTA1) or of DHFRΔ112 (encoding the first 75 amino acids of mouse dihydrofolate reductase [DHFR]), the corresponding sequence of pSPCTA1 or of pGEM4-DHFR (gift of Dr. T. Langer, Ludwig-Maximilians-Universität, Munich, Germany) including the SP6 promoter was amplified by PCR and used as a template for in vitro transcription. The 3′-primer used for the generation of CTA1Δ137 coded for an additional methionine. Therefore, both CTA1Δ137 and DHFRΔ112 contained four methionine residues allowing the same level of labeling. The cDNA coding for α1-AT was obtained from ATCC (no. 61594) and subcloned into the vector pSVSport1. All templates were transcribed with the AmpliScribe SP6 High Yield Transcription Kit (Epicentre Technologies).

Export Assay

High salt–washed microsomes were prepared from porcine pancreas (Walter and Blobel, 1983). The purity and integrity of the preparation was verified by determining the absorbance at 260 and 280 nm (OD_{260}/$OD_{280} > 1.8$) and by electron microscope examination (see below). The microsomes were loaded by cotranslational translocation of in vitro translated CTA1. The in vitro translation reaction contained 50% reticulocyte lysate (Promega), 0.3 µCi/µl [^{35}S]methionine, 0.05 µg/µl mRNA, 0.4 U/µl RNasin, and 0.1 eq/µl microsomes (for definition of eq, see Walter and Blobel, 1983). Translation was performed for 10 min at 30°C and stopped by the addition of puromycin (2 mM final concentration) for 5 min. The microsomes were separated from the lysate by centrifugation (9,000 g, 10 min, 4°C), resuspended in cold PBS, and divided into aliquots (each corresponding to a 10-µl translation reaction). The export reaction was started by the addition of twofold concentrated export buffer plus ATP (4 mM $MgCl_2$, 2 mM ATP, 20 mM creatine phosphate, 400 µg/ml creatine kinase in PBS, pH 7.5) or twofold concentrated export buffer minus ATP (4 mM $MgCl_2$, 50 mM glucose, 60 U/ml hexokinase in PBS, pH 7.5), and incubated at 30°C. At the end of the export period, the microsomes were reisolated by centrifugation. The microsomal pellet and the supernatant were analyzed by SDS-PAGE (Schägger and von Jagow, 1987) using a 12% separating gel and autoradiography. To analyze export in the presence of cytosol, microsomes loaded with CTA1 were resuspended in 50% reticulocyte lysate in export buffer plus ATP. At the end of the export period, the microsomes were separated by centrifugation, and CTA1 present in the supernatant was isolated by immunoprecipitation with anti–cholera toxin antiserum (Guildhay).

For quantitative analysis, the gels were exposed on a phosphor storage imager (Fuji BAS1000). For each time point, the fraction of CTA1 found in the supernatant (fs) was calculated as percentage of total CTA1 present in the pellet and the supernatant. The export at a given time point $[e_{t(x)}]$ was determined by subtracting fs at 0 min export from fs at the given time ($e_{t(x)} = fs_{t(x)} - fs_{t(0)}$).

Protease Protection Assays

Microsomes were loaded with CTA1 and isolated at the end of translation by centrifugation. The microsomes were resuspended in PBS containing the indicated concentration of trypsin, and where indicated, 1% Triton X (TX)-100. After incubation for 30 min on ice, trypsin activity was stopped by the addition of phenylmethylsulfonylfluoride to 1 mM and the products were analyzed by SDS-PAGE and autoradiography.

Protease Resistance Assay

After translation, the microsomes were isolated by centrifugation. One aliquot was boiled for 5 min in 0.5% SDS, the other aliquot was kept on ice. Both aliquots were diluted with 5 vol of PBS containing 1% TX-100, treated with the indicated concentrations of trypsin or proteinase K, respectively, for 30 min on ice and further processed as described above.

Coimmunoprecipitation of CTA1 with the Sec61p Complex

Translation of CTA1 (15 min) or α1-AT (30 min) was stopped by the addition of 2 mM puromycin for 5 min, and the microsomes were isolated by

centrifugation, resuspended in export buffer minus ATP, and chased for up to 1 h at 30°C. At the end of the chase, 5 vol of 0.5 M potassium acetate, pH 7.5, 5 mM MgCl$_2$, 0.2 mM GTP, 1 mM puromycin were added and the microsomes were incubated for further 15 min at 30°C. The samples were adjusted to 0.75% deoxyBigCHAP (Calbiochem) and incubated for 20 min on ice. After clearing the lysate (18,000 g, 15 min, 4°C) BSA was added to 0.2%. CTA1, α1-AT, or the Sec61p complex were immunoprecipitated for 3 h at 4°C using goat anti–cholera toxin antiserum, rabbit anti–α1-AT antiserum (Calbiochem), or rabbit anti-Sec61β antiserum (High et al., 1993), which had been bound to protein G–agarose. Controls were treated with protein G–agarose only. Washings were done with 0.5 M potassium acetate, pH 7.5, 0.25% deoxyBigCHAP. The samples were analyzed by SDS-PAGE. For α1-AT, a 10% separating gel was used (Laemmli, 1970). Immunoprecipitated bands were quantified using a phosphor storage imager.

Isolation of Ribosome Nascent Chain Complexes

CTA1Δ137-mRNA or DHFRΔ112-mRNA was translated for 10 min in a reticulocyte lysate. Translation was stopped by cycloheximide (0.3 mM) and the reaction diluted with the same volume of 1 M potassium acetate, 50 mM Tris-Cl, pH 7.5, 250 mM sucrose, 5 mM magnesium acetate. Ribosome nascent chain complexes (RNCs) were isolated by centrifugation at 40,000 g for 1 h and resuspended in 2× binding buffer (1 mM GTP, 8 μg/ml aprotinin, 20 μM E-64 in PBS).

Blocking Sec61p Complexes by RNCs

For blocking the Sec61p complexes with isolated RNCs, the microsomes (0.025 eq/μl) were loaded with CTA1, reisolated, and resuspended in export buffer plus ATP. One half of the microsomes was incubated for 10 min at 30°C with a saturating amount of CTA1Δ137, the other half with DHFRΔ112. The microsomes were used for an export reaction in the presence of ATP as described above with the only exception being that RNase A was added to 300 μg/ml for 5 min at the end of the export period and before centrifugation.

The amount of CTA1Δ137 necessary to saturate the available Sec61p complexes was determined by incubating in binding buffer as above 0.25 eq microsomes with increasing amounts of CTA1Δ137. After binding, 1 vol of 1 M potassium acetate, 4 mM MgCl$_2$, 4 mM DTT, 80 mM Hepes, pH 7.5, was added and the microsomes were incubated for 15 min on ice. After centrifugation, the microsomal pellet and the supernatant were analyzed by SDS-PAGE and autoradiography. CTA1Δ137 isolated from a 60-μl translation reaction was sufficient for saturation. In the competition experiments RNCs isolated from 100 μl translation reactions were used.

Preparation of Lumenal Proteins

For the preparation of lumenal microsomal proteins, high salt EDTA-washed microsomes were suspended at 1 eq/μl in 25 mM Tris-Cl, pH 7.5, 250 mM sucrose, 23 mM N-octylglucoside (Calbiochem), and incubated for 30 min on ice. After centrifugation in a Ti55.2 rotor (55,000 rpm, 1 h), the supernatant containing the lumenal proteins was precipitated by ammonium sulfate (90% saturation). The precipitate was desalted using EconoPac 10 DG columns (Bio-Rad) equilibrated in 25 mM Tris-Cl, pH 7.5, 250 mM sucrose. To remove the detergent, each milliliter of extract was transferred to 200 mg Bio-Beads SM-2 (Bio-Rad) and incubated for 2 h at 4°C with one change of Bio-Beads. The fluid phase is referred to as lumenal proteins.

Extraction and Resubstitution of Microsomes

For the resubstitution of lumenal proteins and the reconstitution of CTA1-containing microsomes, the method of Görlich and Rapoport to prepare proteoliposomes from microsomal membrane extracts was adapted (Görlich and Rapoport, 1993). Microsomes (1 eq/μl) were permeabilized in 50 mM Hepes-KOH, pH 7.8, 350 mM potassium acetate, 12 mM MgCl$_2$, 5 mM β-mercaptoethanol, 15% glycerol, 0.24% deoxyBigCHAP for 30 min on ice. 100-μl portions were added to 40 mg Bio-Beads SM-2 and incubated for 3–4 h on a rotating device at 4°C. The fluid phase was separated and diluted with 10 vol of cold Tris-buffered saline, pH 7.5. The resealed microsomes were collected by centrifugation (18,300 g, 15 min, 4°C) (extracted microsomes). For resubstitution with lumenal proteins, 100 eq extracted microsomes were resuspended in 100 μl buffer (25 mM Tris-Cl, pH 7.5, 250 mM sucrose, 0.24% deoxyBigCHAP) containing 2 eq/μl lumenal proteins. For reconstituting CTA1-containing microsomes, ex-

tracted microsomes were incubated with 0.2 mg/ml CTA (Calbiochem) alone or with 0.2 mg/ml CTA and 2 eq/μl lumenal proteins. After a 30-min incubation at 4°C, the detergent was removed and the microsomes collected as above. Mock-treated microsomes were treated identically to extracted microsomes but the detergent was omitted. The microsomes were resuspended at 1 eq/μl in 25 mM Tris-Cl, pH 7.5, 250 mM sucrose. If not stated otherwise, 1 eq was used per 10-μl translation reaction and 1.6 eq for the corresponding nonradioactive assays.

For the analysis of the extraction-resubstitution, identical amounts of the microsomes were separated by SDS-PAGE (Laemmli, 1970) and heavy chain binding protein (BiP) was detected by immunoblot using a goat anti-BiP antiserum (Santa Cruz Biotechnology).

EM of Microsomal Preparations

Microsomes were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.6, postfixed in 1% OsO$_4$ in H$_2$O, stained en bloc with 2% uranyl acetate in H$_2$O, dehydrated, and embedded in epon. Sections were mounted on formvar-coated grids and viewed in a Philips electron microscope CM120. Care was taken to search in all parts of the microsomal pellets for possible contaminations. Only fractions with highest microsomal purity were used for the experiments (data not shown).

Results

The aim of this study was to elucidate the mode of export of CTA1 from the lumen of the ER to the cytosol. For this purpose we established a cell-free export system using translocation-competent microsomes as an in vitro substitute for the ER. The microsomes were either loaded with in vitro synthesized CTA1 by cotranslational import of the protein, or reconstituted with CTA1 purified from *V. cholerae*, and the export of CTA1 from the microsomes was analyzed.

Import and Folding of CTA1 in Microsomes

CTA1 was in vitro synthesized in a reticulocyte lysate and cotranslationally imported into the lumen of microsomes. Upon import into the microsomes, the signal peptide of the precursor of CTA1 was removed, resulting in a higher electrophoretic mobility of CTA1 as compared with pre-CTA1 (Fig. 1, lanes 1 and 2). When the microsomes were separated from the reticulocyte lysate by centrifugation, CTA1 was found in the microsomal pellet (Fig. 1, lane 3). To demonstrate that CTA1 was not simply attached to the outside of the microsomal membranes, the microsomes were treated with trypsin after translation. CTA1 was completely degraded in the presence of detergent but protected from degradation in the absence of detergent, demonstrating its complete transfer across the microsomal membrane (Fig. 1, lanes 3–5). To use glycosylation as additional evidence for import of CTA1 into the microsomes, a mutant proinsulin containing an *N*-glycosylation site (Schmitz et al., 1995) was added to the COOH terminus of CTA1, which itself is not glycosylated. The electrophoretic mobility of this fusion protein (CTA1Ins) was increased when a competitive inhibitor of the lumenally active oligosaccharyltransferase was added (Fig. 1, lanes 9 and 10), indicating that it was glycosylated after import into the microsomes. Similar to CTA1, CTA1Ins was protected from trypsin added to the outside of the microsomes (Fig. 1, lanes 6–8), again demonstrating its complete import into the microsomes.

After import into the microsomes, CTA1 might remain largely unfolded or misfolded or it might fold into a compact conformation. To discriminate between these possi-
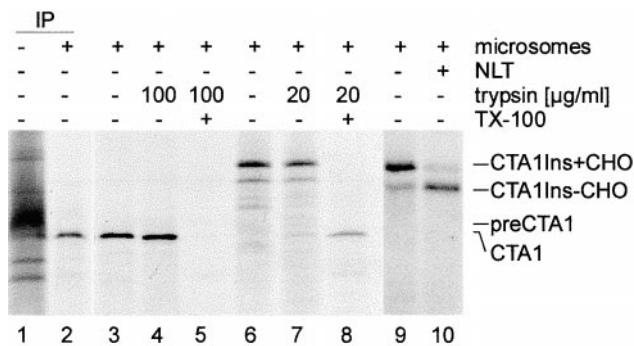
Figure 1. Import of CTA1 into microsomes. CTA1 (lanes 1–5) and CTA1Ins (lanes 6–10) were translated in a reticulocyte lysate. (Lanes 1 and 2) After translation in the absence or presence of microsomes, CTA1 and its precursor (preCTA1) were immunoprecipitated. (Lanes 3–8) After translation, the microsomes were isolated by centrifugation and treated with trypsin or trypsin and TX-100. (Lanes 9 and 10) Glycosylation of CTA1Ins was inhibited by the addition of the tripeptide *N*-acetyl-NLT-NH$_2$ (100 μM). The proteins were analyzed by SDS-PAGE and autoradiography.

Figure 2. Folding of CTA1 inside the microsomes. CTA1 was translated for 10 min and the microsomes were isolated by centrifugation. One half was boiled for 5 min in 0.5% SDS, the other half was kept on ice. Both samples were diluted with 5 vol PBS containing 1% TX-100, divided into aliquots, incubated for 30 min on ice with the indicated concentrations of trypsin (upper panel) or proteinase K (lower panel), and analyzed by SDS-PAGE and autoradiography.

bilities CTA1 was treated with proteases under nondenaturing or denaturing conditions. In the first case, the microsomes were lysed with TX-100, whereas in the latter case the microsomes were first boiled in SDS and then the SDS was quenched by the addition of TX-100. Digestion of these samples with increasing concentrations of trypsin showed that CTA1 lost its resistance against proteolysis upon denaturation (Fig. 2). The same result was obtained using the more promiscuous protease proteinase K. The finding that CTA1 became more sensitive to proteolysis upon denaturation argued against the possibility that the in vitro synthesized and imported CTA1 remained unfolded or was grossly misfolded, but indicated that it had folded into a compact conformation inside the microsomes. This assumption is supported by the finding that treatment of CTA1Ins with a low concentration of trypsin resulted in the degradation of the proinsulin moiety, whereas the CTA1 moiety remained stable (Fig. 1, lane 8). In contrast to CTA1, the proinsulin moiety, which contains three disulfide bonds, cannot fold correctly in the reducing environment of the in vitro translation system. Thus, by the criteria of signal peptide cleavage, glycosylation, protease protection, and protease resistance, CTA1 was completely imported into the microsomes where it folded into a compact conformation.

Export of CTA1 from Microsomes

The export assay is composed of two steps: first, the loading of the microsomes with CTA1 as described above; and second, the actual export from the lumen of the microsomes into the surrounding buffer. After the termination of translation by the addition of puromycin, the microsomes were separated from the reticulocyte lysate by centrifugation, resuspended in buffer with or without ATP, and divided into aliquots, which were incubated for increasing chase periods. At the end of the chase period the microsomes were separated from the buffer by centrif-
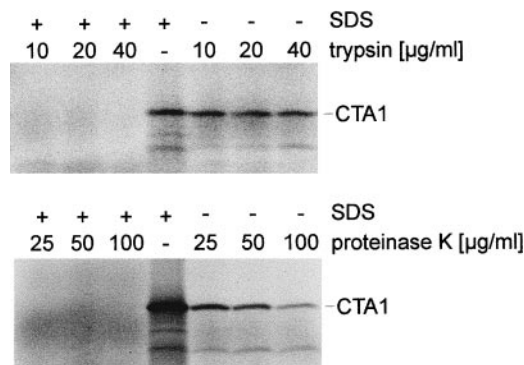
ugation and both fractions analyzed by SDS-PAGE and autoradiography. Whereas in the absence of ATP CTA1 was barely detectable in the buffer, a time-dependent increase of CTA1 in the buffer was found in the presence of an ATP regenerating system (Fig. 3 a). The exported CTA1 had the same electrophoretic mobility as the CTA1 present in the microsomes, indicating that its signal peptide had been cleaved after import into and before export from the microsomes. In addition, the identical molecular weight of microsomal and exported CTA1 indicated that exported CTA1 was not ubiquitinated. In agreement with this observation, no ubiquitinated CTA1 was found by immunoblot analysis using an antiubiquitin antibody (data not shown).

To calculate export rates, the fraction of CTA1 present in the buffer was determined for each time point. From this value the amount of CTA1 found in the buffer at 0 min export, which was in average 5.3% ($\pm$ 3.4% SD; $n = 21$), was subtracted (Fig. 3 b). The mean export rate of reactions performed in the presence of ATP was 17.8%/h ($\pm$ 5.8% SD), whereas export in the absence of ATP was 1.9%/h ($\pm$ 0.8% SD). The export was not significantly changed by the addition of cytosol (22.5%/h $\pm$ 5.1% SD). The kinetics of export differed between different microsomal preparations in that export was more pronounced during the first hour in some preparations and during the second hour in other preparations. The reason for this difference is not yet clear.

To exclude the possibility that export of CTA1 from the microsomes occurred because the in vitro synthesized CTA1 had not been completely released from the lumenal side of the Sec61p complex after its import, a different approach to load the microsomes with CTA1 was used. For this purpose, microsomes were reconstituted with CTA1, which had been isolated from *V. cholerae*. The loss of lumenal ER proteins during the reconstitution was prevented by adding concentrated lumenal ER proteins to the reconstitution buffer. The reconstituted CTA1-containing microsomes were used for an export assay as described
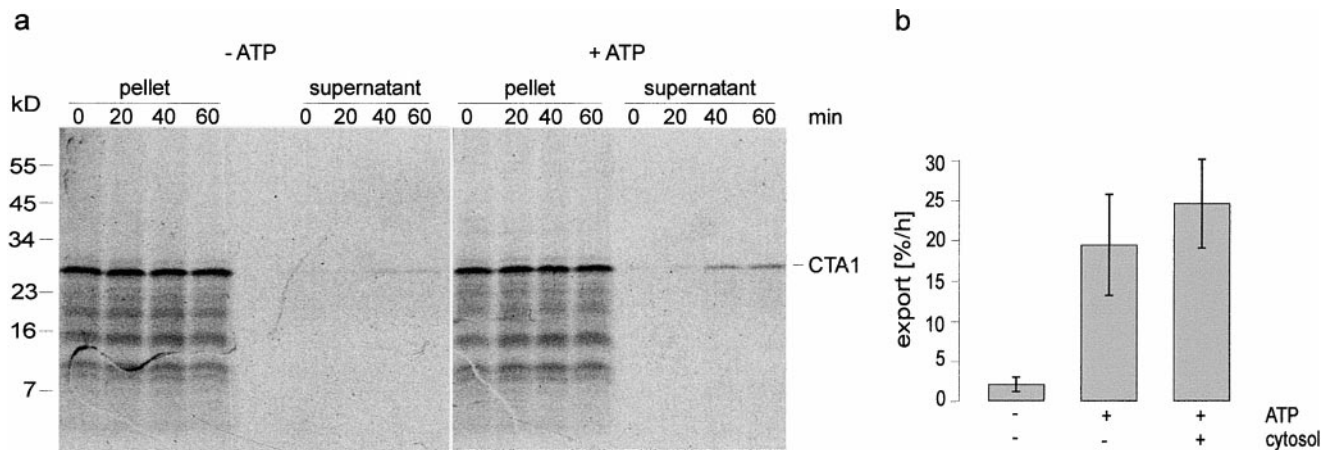
Figure 3. Export of CTA1 from microsomes. (a) After translation (10 min) of CTA1 in a single reaction, the microsomes were reisolated by centrifugation, resuspended in PBS, and divided into aliquots. The export reaction was started by the addition of export buffer with or without ATP. At the end of the chase period the microsomes were reisolated by centrifugation. The presence of CTA1 in the microsomal pellet and the supernatant was analyzed by SDS-PAGE and autoradiography. (b) Statistical analysis of export reactions performed for 1 h without ATP ($n = 5$), with ATP ($n = 13$), or with ATP and cytosol ($n = 5$). For the calculation of export rates, the fraction of CTA1 found in the supernatant at 0 min chase was subtracted. Bars represent SD.

above. CTA1 was detected by immunoblot using an anti–cholera toxin antiserum. As with the biosynthetically loaded microsomes, CTA1 was exported from the reconstituted microsomes in an ATP-dependent manner (Fig. 4 a). To demonstrate that the reconstituted microsomes contained CTA1 in their lumen and not only attached to the outside of the membrane, a protease protection analysis was performed (Fig. 4 b). CTA1, which was degraded in the presence of detergent, was protected from degradation in the absence of detergent. The finding of export of

Figure 4. Export from reconstituted microsomes. (a) Microsomes reconstituted with CTA1 and lumenal ER proteins (see Materials and Methods) were resuspended in export buffer with or without ATP and used for an export reaction as described in Fig. 3 a. CTA1 was detected by immunoblot using an anti–cholera toxin antiserum. The export rates per hour were <1% in the absence of ATP and 9% in the presence of ATP, respectively. 10 ng of the CTA1 used for the reconstitution was loaded as a standard (st). (b) Reconstituted microsomes were treated with 100 μg/ml trypsin in the absence or presence of TX-100. CTA1 was detected by immunoblot.

CTA1 from reconstituted microsomes demonstrated that export of CTA1 did not require the persistent interaction of CTA1 with the translocon after import. As folded, enzymatically active CTA1 was used for the reconstitution of the microsomes. This finding also argued against the possibility that the in vitro translated and imported CTA1 was exported, because it could not fold correctly and thus simply represented a misfolded protein.

For the reconstitution of the CTA1-containing microsomes, high salt–washed microsomes, lumenal ER proteins prepared from high salt/EDTA–washed microsomes, and CTA1 purified from *V. cholerae* were used. No cytosol was added before or during export. Both export from the reconstituted microsomes and the observation that export was not stimulated by the addition of cytosol (see Fig. 3 b) supported the conclusion that cytosolic proteins were not required for the export of CTA1 from microsomes.
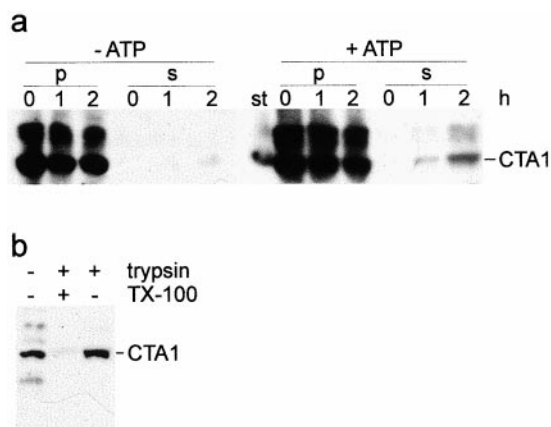
Export of CTA1 Is Mediated by the Sec61p Complex

To analyze whether export of CTA1 was mediated by the Sec61p complex, the physical interaction of CTA1 with the Sec61p complex was examined. The import of the in vitro translated CTA1 was stopped by the addition of puromycin, which induces the release of unfinished nascent chains from the ribosome and from the Sec61p complex (Görlich et al., 1992). The microsomes were reisolated and chased in export buffer without ATP to stabilize the export intermediate which was assumed to interact with the Sec61p complex. At the end of the chase, the microsomes were stripped of remaining ribosomes by incubation in high salt/puromycin buffer and lysed with deoxyBigCHAP, which leaves the Sec61p complex intact (Görlich et al., 1992). The Sec61p complex was immunoprecipitated with an anti-Sec61β antiserum under high salt conditions (Fig. 5). CTA1 was coimmunoprecipitated with the Sec61p complex, indicating their physical interaction. The nonexported secretory protein α1-AT was not coimmunoprecip-
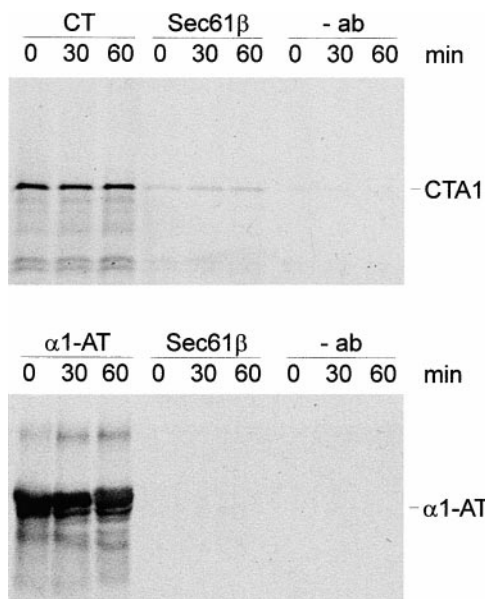
Figure 5. Association of CTA1 with the Sec61p complex during export. Translation of CTA1 or α1-AT was terminated by the addition of puromycin and the microsomes were reisolated and resuspended in export buffer minus ATP. After a chase for the indicated time, the microsomes were incubated in puromycin/high salt buffer and lysed with 0.75% deoxyBigCHAP. CTA1, α1-AT, and the Sec61p complex were immunoprecipitated with anti–cholera toxin antiserum (CT), anti–α1-AT antiserum, or anti–Sec61β antiserum, respectively. For the immunoprecipitation with the anti-Sec61β antiserum and for the controls without antibody (−ab) the threefold amount of microsomes was used as compared with the anti–cholera toxin and anti–α1-AT immunoprecipitations. During the chase, the fraction of CTA1 coimmunoprecipitated with the Sec61p complex increased from 2 to 4%.

itated with the Sec61p complex, indicating the specificity of the interaction between CTA1 and the Sec61p complex. In three independent experiments, the fraction of coimmunoprecipitated CTA1 consistently increased during the chase period by a factor between 1.5 and 2, indicating that CTA1 had left the Sec61p complex after import and reassociated with it during the export. It has been suggested that Sec61β may, under certain conditions, exist in the ER membrane as an individual protein, i.e., not integrated into the Sec61p complex (Kalies et al., 1998). Therefore, it cannot be entirely excluded that CTA1 was associated with this fraction of Sec61β.

To establish that the Sec61p complex was functionally necessary for the export of CTA1, a block of the Sec61p complex was used. It is well established that the nascent chain is released and the ribosome dissociates from the Sec61p complex when the stop codon of a translated mRNA molecule is reached. This termination reaction can be prevented by the translation of mRNA lacking the stop codon, resulting in a tight complex between the ribosome, the Sec61p complex, and the truncated nascent chain that is arrested in transit through the Sec61p complex.

Microsomes were loaded with CTA1 by in vitro translation. The reisolated microsomes were then incubated with truncated nascent chains that had been isolated as RNCs
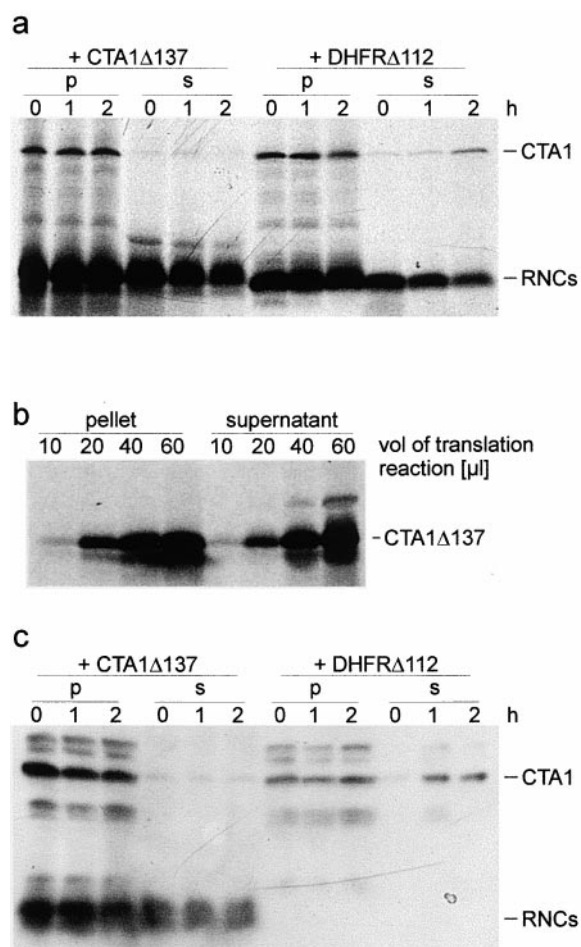
Figure 6. Sec61p complex–mediated export of CTA1. (a) Microsomes were loaded with CTA1 by in vitro translation, isolated by centrifugation, and resuspended in export buffer containing ATP. One half of the microsomes was incubated for 10 min at 30°C with saturating amounts (corresponding to a 100-μl translation reaction; see below) of CTA1Δ137, the other half with DHFRΔ112, which did not contain a signal peptide and was used as a control. After binding of the RNCs, the microsomes were used for an export reaction as described in Fig. 3 a. CTA1, CTAΔ137, and DHFRΔ112 were detected by autoradiography. Export rates per hour were <1% in the presence of CTA1Δ137 and 11% in the presence of DHFRΔ112, respectively. (b) Microsomes were incubated with increasing amounts of CTA1Δ137. After binding, the microsomes were high salt–washed to remove unspecifically bound RNCs. The reactions were separated by centrifugation in a pellet fraction containing the Sec61p complex–bound RNCs and a supernatant fraction containing the unbound RNCs. The amount of CTA1Δ137 isolated from a 60-μl translation reaction was sufficient to saturate the microsomes. (c) Microsomes reconstituted with purified CTA1 and lumenal ER proteins were incubated with RNCs and used for an export reaction exactly as described in a. CTA1 and CTA1Δ137 were detected by immunoblot using an anti–cholera toxin antiserum. Due to the immunodetection DHFRΔ112 is not visible. Export rates per hour were <1% in the presence of CTA1Δ137 and 16% in the presence of DHFRΔ112, respectively.

from a separate translation reaction. The RNCs were generated by translation of CTA1Δ137, which corresponds to the first 75 amino acids of CTA1, or of DHFRΔ112, which corresponds to the first 75 amino acid of DHFR. As

DHFR does not contain a signal peptide and is not imported into microsomes, it was used as a control to demonstrate that only signal peptide containing RNCs inhibit export of CTA1. When export of CTA1 was performed in the presence of saturating amounts of CTA1Δ137, the export of CTA1 was completely inhibited. In contrast, export occurred unhindered when DHFRΔ112 was used for competition (Fig. 6 a). In this type of experiment the export was consistently more pronounced in the second hour of chase. The saturation of all Sec61p complexes with CTA1Δ137 was verified by determining the maximal amount of RNCs bound to the microsomes in a high salt–resistant manner, indicating their insertion into the Sec61p complex (Fig. 6 b). RNCs isolated from a 60-μl translation reaction were sufficient to saturate all Sec61p complexes available in 0.25 eq microsomes. In the competition experiments, RNCs isolated from 100-μl translation reactions were used.

To demonstrate that the export of CTA1 was also inhibited by CTA1Δ137 under conditions where persistent interaction of CTA1 with the Sec61p complex after import could be excluded, microsomes reconstituted with purified CTA1 and lumenal ER proteins were used. In addition, under these experimental conditions export of CTA1 was inhibited by the addition of CTA1Δ137 but not by DHFRΔ112 (Fig. 6 c).

The competition data demonstrated that the export of CTA1 was inhibited by functionally blocking the Sec61p complex. Together with the coimmunoprecipitation data demonstrating association of CTA1 with the Sec61p complex during export, these results indicated that the Sec61p complex mediated the export of CTA1 across the microsomal membrane.

Export of CTA1 Requires Lumenal ER Proteins

To test whether in addition to the Sec61p complex lumenal ER proteins were required for export of CTA1, microsomes were depleted of their lumenal proteins by detergent extraction (Fig. 7 a). In contrast to mock-treated microsomes, the extracted microsomes that were still able to import CTA1 showed no export of CTA1. The export of CTA1 was recovered after resubstituting the extracted microsomes with lumenal ER proteins. The efficiency of the depletion and resubstitution was verified by immunodetection of the ER-resident chaperone BiP (Fig. 7 b).

The inability of the extracted microsomes to export CTA1 could be caused indirectly by their inability to support the folding of the newly imported CTA1, which due to aggregation could be unavailable for export. To exclude this possibility, microsomes were reconstituted with purified, folded, and enzymatically active CTA1 either in the presence or absence of lumenal ER proteins. Also, under these conditions export occurred only in the presence of lumenal ER proteins (Fig. 7 c). The finding that depletion of lumenal ER proteins resulted in a reversible export inhibition demonstrated that lumenal ER proteins were required for export of CTA1.
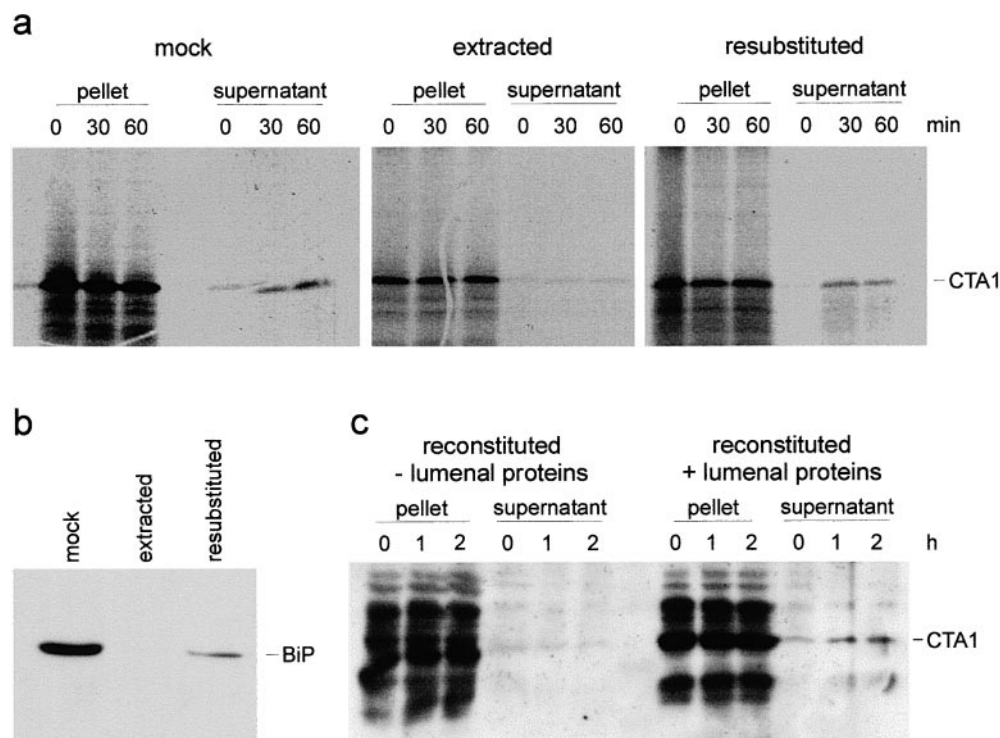

Figure 7. Dependence of CTA1 export on lumenal ER proteins. Microsomes were depleted of their lumenal proteins by extraction with deoxyBigCHAP or were, after extraction, resubstituted with lumenal ER proteins. (a) Mock-treated, extracted, or resubstituted microsomes were loaded with CTA1 by in vitro translation. Export of CTA1 from the different microsomes was analyzed as described in Fig. 3 a. The export rates per hour were 10, 0, and 11% for the mock-treated, extracted, and resubstituted microsomes, respectively. (b) The efficiency of the extraction and resubstitution was verified by the immunodetection of BiP in equal amounts of mock-treated, extracted, and resubstituted microsomes using an anti-BiP antiserum. (c) Microsomes were reconstituted with CTA1 either in the absence (reconstituted − lumenal proteins) or presence (reconstituted + lumenal proteins) of lumenal ER proteins. Export of CTA1 was analyzed as described in Fig. 3 a, with the exception that CTA1 was detected by immunoblot. The export rates per hour were 0 and 8% in the absence or presence of lumenal proteins, respectively.

Discussion

In this study we show that the bacterial toxin CTA1 is transferred by the Sec61p complex across the ER membrane. Our findings indicate the existence of export mechanisms from the ER to the cytosol independently of proteasomal degradation. As yet, export from the ER to the cytosol has only been described for proteins destined for ER-associated protein degradation. Therefore, our results have implications not only on our current understanding of CTA1 entry into the target cell but also on a more general model of protein translocation across the ER membrane.

Translocation of CTA1 from the ER to the Cytosol

Cholera toxin has been shown to bind to ganglioside GM1, to be endocytosed via noncoated vesicles (Tran et al., 1987; Orlandi and Fishman, 1998), and transported to the Golgi complex where the A and B subunits disassemble (Bastiaens et al., 1996). Whereas the B subunit remains in the Golgi complex, the A subunit can reach the ER (Majoul et al., 1996) via coatomer I–coated vesicles (Majoul et al., 1998), where the disulfide bond between the A1 and A2 subunits is reduced (Majoul et al., 1997; Orlandi, 1997). In spite of intense research on this intriguing sequence of events, it is as yet unknown whether this pathway is a prerequisite for the translocation of CTA1 to the cytosol or only a by-product of its internalization. Indeed, earlier reports exist on the B subunit–mediated insertion of CTA1 into liposomes (Tomasi and Montecucco, 1981; Ribi et al., 1988) and into the membrane of Newcastle disease virus (Wisnieski and Bramhall, 1981). These reports suggested that translocation of CTA1 might be independent of proteins of the target cell but be mediated by a conformational change of the toxin and the consecutive direct penetration through the lipid phase of the membrane. But these studies were restricted to the insertion of CTA1 into membranes, and did not address the transfer across the membranes. In this analysis, the export of CTA1 was ATP-dependent. As CTA1 is not an ATPase, the ATP dependence indicates that CTA1 interacts directly or indirectly with an ATPase during export. The requirement for lumenal ER proteins and for the Sec61p complex supports the view that these proteins of the target cell are essential for export, and that translocation does not occur through the lipid phase of the membrane but through the Sec61p complex–formed channel. The cooperation of these factors is possible only in the ER and therefore, we conclude that translocation of CTA1 in vivo also occurs from the ER. In support of this view, it was shown that brefeldin A (Donta et al., 1993; Nambiar et al., 1993; Orlandi et al., 1993) and mutations in the COOH-terminal ER retrieval signal of the A2 subunit (Lencer et al., 1995) protected cells from intoxication by cholera toxin, suggesting that the ER is functionally involved in the intoxication process.

A Common Mechanism for Export of CTA1 and of Proteins Destined for Degradation?

Export of CTA1 is inhibited by truncated polypeptides that are arrested in transit through the Sec61p complex, demonstrating that the Sec61p complex is essential for the export of CTA1. As misfolded proteins also appear to be exported by the Sec61p complex (Wiertz et al., 1996; Pilon et al., 1997; Plemper et al., 1997; Bebök et al., 1998), a common export machinery with the Sec61p complex as the basic element seems to be used. In addition, ER lumenal proteins are required for export of both CTA1 (this report) and misfolded proteins (Plemper et al., 1997; Brodsky et al., 1999). Thus, not only the basic machinery but also some aspects of the export mechanism may be similar. One key question concerning this mechanism is whether the exported misfolded proteins have completely left the translocon after their import or whether persistent contact with the translocon is required for export to be initiated. Posttranslational reinsertion into the translocon has been inferred from the studies on the degradation of the membrane protein major histocompatibility complex class I heavy chain, which seemed to be folded before export from the ER (Wiertz et al., 1996), and of the soluble protein CPY* (Plemper et al., 1999). However, for both proteins definite proof is lacking that would demonstrate that they had completely left the translocon before their export was initiated. Here, Sec61p complex–dependent export of CTA1 was shown using microsomes reconstituted with purified CTA1. Under these conditions persistent contact of the protein with the translocon after its import can be excluded. Therefore, the export of CTA1 implies that it posttranslationally inserted from the lumenal side into the translocation channel. More generally, export of CTA1 suggests that insertion from the lumenal side into the translocon is possible for a soluble protein after cleavage of the signal peptide, which is the structure recognized by the Sec61p complex during cotranslational (Jungnickel and Rapoport, 1995; Mothes et al., 1998) and posttranslational (Plath et al., 1998) import of polypeptides into the ER.

The observations presented here suggest that the translocation of both CTA1 and of proteins destined for degradation is mediated by the Sec61p complex, and may be based on a similar basic mechanism. However, major differences exist that indicate that the translocation mechanisms for both groups of proteins are not identical.

Differences in the Translocation of CTA1 and of Degradation Substrates

A tight coupling of export and degradation has been suggested by several studies. The integral membrane proteins CD4 (Schubert et al., 1998), CD3-δ (Yang et al., 1998), and a synthetic degradation substrate based on the Sec62 protein (Mayer et al., 1998) accumulated in the membrane-bound form when the proteasome was inhibited. These findings suggested that the catalytic activity of the proteasome was necessary to trigger by an unknown mechanism the dislocation of these proteins from the ER membrane. In contrast, major histocompatibility complex class I heavy chains were exported when proteasomal activity was inhibited (Wiertz et al., 1996), as was pro–α-factor in the presence of cytosol prepared from yeast mutants with defective proteasomes (Werner et al., 1996). The coupling of ubiquitination and export was suggested from the observation that misfolded CPY* accumulated inside the ER when expressed in yeast mutants defective in the ubiquitination at the ER membrane (Biederer et al., 1997).

Similarly, a mutant form of ribophorin I accumulated in the ER when expressed in mammalian cells defective in the ubiquitin-activating enzyme E1 (de Virgilio et al., 1998). Although being independent of ubiquitination, export of pro–α-factor was dependent on the presence of cytosol (McCracken and Brodsky, 1996; Werner et al., 1996).

Here, we show that export of CTA1 requires lumenal ER proteins but occurs in the absence of cytosol and without apparent ubiquitination. Under the experimental conditions used here, active proteasomes, as identified by their ability to support the degradation of a mutant form of α1-AT (Qu et al., 1996), were not detected (data not shown). Most probably, this lack of active proteasomes is due to the presence of hemin in the reticulocyte lysate acting as a potent inhibitor of proteasomes (Hough et al., 1986). Thus, the export of CTA1 seems to be independent of the cytosolic factors required for the export of proteins destined for degradation, but seems to rely entirely on factors residing in the ER lumen and the ER membrane.

The proteasome, ubiquitination, and cytosolic chaperones are discussed to regulate the export of proteins destined for degradation (Brodsky and McCracken, 1999). As none of these proteins appears to be required for the export of CTA1, the question arises as to how its export is regulated. Protein import by the Sec61p complex needs the cooperation of the complex with other constituents such as the ribosome during cotranslational import, or the ER-resident chaperone BiP during posttranslational import in yeast (Sanders et al., 1992; Matlack et al., 1999). BiP binds to stretches of exposed hydrophobic amino acids (Flynn et al., 1991; Blond-Elguindi et al., 1993) and observations indicate that this binding affects the degradation of secretory proteins in mammalian cells (Knittler and Haas, 1992; Schmitz et al., 1995). Genetic screens implicated BiP to participate in the export of CPY* (Plemper et al., 1997) and pro–α-factor (Brodsky et al., 1999) in yeast. But it is unclear whether BiP provides by its ATPase activity the driving force for the export of one of these proteins or is required for the unfolding of the proteins. BiP might also be involved in the gating of the translocon for protein export as it was shown to seal nontranslocating translocons (Hamman et al., 1998). We found that the export of CTA1 was dependent on ATP and lumenal ER proteins but independent of cytosolic proteins. Our findings support the view that a basic level of regulation of protein export from the ER is independent of cytosolic factors but is achieved by the cooperation of the Sec61p complex with an ER-resident ATPase.

References

Bastiaens, P.I., I.V. Majoul, P.J. Verveer, H.D. Söling, and T.M. Jovin. 1996. Imaging the intracellular trafficking and state of the AB5 quaternary structure of cholera toxin. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:4246–4253.

Bebök, Z., C. Mazzochi, S.A. King, J.S. Hong, and E.J. Sorscher. 1998. The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum to the proteasome includes Sec61beta and a cytosolic, deglycosylated intermediary. *J. Biol. Chem.* 273:29873–29878.

Beckmann, R., D. Bubeck, R. Grassucci, P. Penczek, A. Verschoor, G. Blobel, and J. Frank. 1997. Alignment of conduits for the nascent polypeptide chain in the ribosome-Sec61 complex. *Science.* 278:2123–2126.

Biederer, T., C. Volkwein, and T. Sommer. 1997. Role of Cue1p in ubiquitination and degradation at the ER surface. *Science.* 278:1806–1809.

Blond-Elguindi, S., S.E. Cwirla, W.J. Dower, R.J. Lipshutz, S.R. Sprang, J.F. Sambrook, and M.J. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell.* 75:717–728.

Brodsky, J.L., and A.A. McCracken. 1999. ER protein quality control and proteasome-mediated protein degradation. *Semin. Cell Dev. Biol.* 10:507–513.

Brodsky, J.L., E.D. Werner, M.E. Dubas, J.L. Goeckeler, K.B. Kruse, and A.A. McCracken. 1999. The requirement for molecular chaperones during endoplasmic reticulum-associated protein degradation demonstrates that protein export and import are mechanistically distinct. *J. Biol. Chem.* 274:3453–3460.

Crowley, K.S., S. Liao, V.E. Worrell, G.D. Reinhart, and A.E. Johnson. 1994. Secretory proteins move through the endoplasmic reticulum membrane via an aqueous, gated pore. *Cell.* 78:461–471.

de Virgilio, M., H. Weninger, and N.E. Ivessa. 1998. Ubiquitination is required for the retro-translocation of a short-lived luminal endoplasmic reticulum glycoprotein to the cytosol for degradation by the proteasome. *J. Biol. Chem.* 273:9734–9743.

Donta, S.T., S. Beristain, and T.K. Tomicic. 1993. Inhibition of heat-labile cholera and *Escherichia coli* enterotoxins by brefeldin A. *Infect. Immun.* 61:3282–3286.

Flynn, G.C., J. Pohl, M.T. Flocco, and J.E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature.* 353:726–730.

Görlich, D., and T.A. Rapoport. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. *Cell.* 75:615–630.

Görlich, D., S. Prehn, E. Hartmann, K.U. Kalies, and T.A. Rapoport. 1992. A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. *Cell.* 71:489–503.

Hamman, B.D., L.M. Hendershot, and A.E. Johnson. 1998. BiP maintains the permeability barrier of the ER membrane by sealing the lumenal end of the translocon pore before and early in translocation. *Cell.* 92:747–758.

Hanein, D., K.E. Matlack, B. Jungnickel, K. Plath, K.U. Kalies, K.R. Miller, T.A. Rapoport, and C.W. Akey. 1996. Oligomeric rings of the Sec61p complex induced by ligands required for protein translocation. *Cell.* 87:721–732.

Hazes, B., and R.J. Read. 1997. Accumulating evidence suggests that several AB-toxins subvert the endoplasmic reticulum-associated protein degradation pathway to enter target cells. *Biochemistry.* 36:11051–11054.

High, S., B. Martoglio, D. Görlich, S.S. Andersen, A.J. Ashford, A. Giner, E. Hartmann, S. Prehn, T.A. Rapoport, B. Dobberstein, and J. Brunner. 1993. Site-specific photocross-linking reveals that Sec61p and TRAM contact different regions of a membrane-inserted signal sequence. *J. Biol. Chem.* 268:26745–26751.

Hiller, M.M., A. Finger, M. Schweiger, and D.H. Wolf. 1996. ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science.* 273:1725–1728.

Hough, R., G. Pratt, and M. Rechsteiner. 1986. Ubiquitin-lysozyme conjugates. Identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates. *J. Biol. Chem.* 261:2400–2408.

Jensen, T.J., M.A. Loo, S. Pind, D.B. Williams, A.L. Goldberg, and J.R. Riordan. 1995. Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell.* 83:129–135.

Johannes, L., D. Tenza, C. Antony, and B. Goud. 1997. Retrograde transport of KDEL-bearing B-fragment of Shiga toxin. *J. Biol. Chem.* 272:19554–19561.

Johnson, A.E. 1997. Protein translocation at the ER membrane: a complex process becomes more so. *Trends Cell Biol.* 7:90–95.

Jungnickel, B., and T.A. Rapoport. 1995. A posttargeting signal sequence recognition event in the endoplasmic reticulum membrane. *Cell.* 82:261–270.

Kalies, K.U., T.A. Rapoport, and E. Hartmann. 1998. The beta subunit of the Sec61 complex facilitates cotranslational protein transport and interacts with the signal peptidase during translocation. *J. Cell Biol.* 141:887–894.

Knittler, M.R., and I.G. Haas. 1992. Interaction of BiP with newly synthesized immunoglobulin light chain molecules: cycles of sequential binding and release. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:1573–1581.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227:680–685.

Lencer, W.I., C. Constable, S. Moe, M.G. Jobling, H.M. Webb, S. Ruston, J.L. Madara, T.R. Hirst, and R.K. Holmes. 1995. Targeting of cholera toxin and *Escherichia coli* heat labile toxin in polarized epithelia: role of COOH-terminal KDEL. *J. Cell Biol.* 131:951–962.

Lencer, W.I., T.R. Hirst, and R.K. Holmes. 1999. Membrane traffic and the cellular uptake of cholera toxin. *Biochim. Biophys. Acta.* 1450:177–190.

Lord, J.M., and L.M. Roberts. 1998. Toxin entry: retrograde transport through the secretory pathway. *J. Cell Biol.* 140:733–736.

Majoul, I.V., P.I. Bastiaens, and H.D. Söling. 1996. Transport of an external Lys-Asp-Glu-Leu (KDEL) protein from the plasma membrane to the endoplasmic reticulum: studies with cholera toxin in Vero cells. *J. Cell Biol.* 133: 777–789.

Majoul, I., D. Ferrari, and H.D. Söling. 1997. Reduction of protein disulfide bonds in an oxidizing environment. The disulfide bridge of cholera toxin A-subunit is reduced in the endoplasmic reticulum. *FEBS Lett.* 401:104–108.

Majoul, I., K. Sohn, F.T. Wieland, R. Pepperkok, M. Pizza, J. Hillemann, and H.D. Söling. 1998. KDEL receptor (Erd2p)-mediated retrograde transport of the cholera toxin A subunit from the Golgi involves COPI, p23, and the COOH terminus of Erd2p. *J. Cell Biol.* 143:601–612.

Matlack, K.E., B. Misselwitz, K. Plath, and T.A. Rapoport. 1999. BiP acts as a molecular ratchet during posttranslational transport of prepro-alpha factor across the ER membrane. *Cell.* 97:553–564.

Mayer, T.U., T. Braun, and S. Jentsch. 1998. Role of the proteasome in membrane extraction of a short-lived ER-transmembrane protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:3251–3257.

McCracken, A.A., and J.L. Brodsky. 1996. Assembly of ER-associated protein degradation in vitro: dependence on cytosol, calnexin, and ATP. *J. Cell Biol.* 132:291–298.

Mekalanos, J.J., D.J. Swartz, G.D. Pearson, N. Harford, F. Groyne, and M. de Wilde. 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. *Nature.* 306:551–557.

Mothes, W., B. Jungnickel, J. Brunner, and T.A. Rapoport. 1998. Signal sequence recognition in cotranslational translocation by protein components of the endoplasmic reticulum membrane. *J. Cell Biol.* 142:355–364.

Nambiar, M.P., T. Oda, C. Chen, Y. Kuwazuru, and H.C. Wu. 1993. Involvement of the Golgi region in the intracellular trafficking of cholera toxin. *J. Cell. Physiol.* 154:222–228.

Olsnes, S., J.O. Moskaug, H. Stenmark, and K. Sandvig. 1988. Diphtheria toxin entry: protein translocation in the reverse direction. *Trends Biochem. Sci.* 13: 348–351.

Orlandi, P.A. 1997. Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J. Biol. Chem.* 272: 4591–4599.

Orlandi, P.A., and P.H. Fishman. 1998. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *J. Cell Biol.* 141:905–915.

Orlandi, P.A., P.K. Curran, and P.H. Fishman. 1993. Brefeldin A blocks the response of cultured cells to cholera toxin. Implications for intracellular trafficking in toxin action. *J. Biol. Chem.* 268:12010–12016.

Pelham, H.R.B., L.M. Roberts, and M. Lord. 1992. Toxin entry: how reversible is the secretory pathway. *Trends Cell Biol.* 2:183–185.

Pilon, M., R. Schekman, and K. Römisch. 1997. Sec61p mediates export of a misfolded secretory protein from the endoplasmic reticulum to the cytosol for degradation. *EMBO J.* 16:4540–4548.

Plath, K., W. Mothes, B.M. Wilkinson, C.J. Stirling, and T.A. Rapoport. 1998. Signal sequence recognition in posttranslational protein transport across the yeast ER membrane. *Cell.* 94:795–807.

Plemper, R.K., and D.H. Wolf. 1999. Retrograde protein translocation: ERAD-ication of secretory proteins in health and disease. *Trends Biochem. Sci.* 24: 266–270.

Plemper, R.K., S. Böhmler, J. Bordallo, T. Sommer, and D.H. Wolf. 1997. Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. *Nature.* 388:891–895.

Plemper, R.K., P.M. Deak, R.T. Otto, and D.H. Wolf. 1999. Re-entering the translocon from the lumenal side of the endoplasmic reticulum. Studies on mutated carboxypeptidase yscY species. *FEBS Lett.* 443:241–245.

Qu, D., J.H. Teckman, S. Omura, and D.H. Perlmutter. 1996. Degradation of a mutant secretory protein, alpha1-antitrypsin Z, in the endoplasmic reticulum requires proteasome activity. *J. Biol. Chem.* 271:22791–22795.

Rapak, A., P.O. Falnes, and S. Olsnes. 1997. Retrograde transport of mutant ricin to the endoplasmic reticulum with subsequent translocation to cytosol. *Proc. Natl. Acad. Sci. USA.* 94:3783–3788.

Rapoport, T.A., B. Jungnickel, and U. Kutay. 1996. Protein transport across the eukaryotic endoplasmic reticulum and bacterial inner membranes. *Annu. Rev. Biochem.* 65:271–303.

Ribi, H.O., D.S. Ludwig, K.L. Mercer, G.K. Schoolnik, and R.D. Kornberg. 1988. Three-dimensional structure of cholera toxin penetrating a lipid membrane. *Science.* 239:1272–1276.

Sanders, S.L., K.M. Whitfield, J.P. Vogel, M.D. Rose, and R.W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell.* 69:353–365.

Sandvig, K., O. Garred, K. Prydz, J.V. Kozlov, S.H. Hansen, and B. van Deurs. 1992. Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature.* 358:510–512.

Schägger, H., and G. von Jagow. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368–379.

Schmitz, A., M. Maintz, T. Kehle, and V. Herzog. 1995. In vivo iodination of a misfolded proinsulin reveals co-localized signals for Bip binding and for degradation in the ER. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:1091–1098.

Schubert, U., L.C. Anton, I. Bacik, J.H. Cox, S. Bour, J.R. Bennink, M. Orlowski, K. Strebel, and J.W. Yewdell. 1998. CD4 glycoprotein degradation induced by human immunodeficiency virus type 1 Vpu protein requires the function of proteasomes and the ubiquitin-conjugating pathway. *J. Virol.* 72: 2280–2288.

Tomasi, M., and C. Montecucco. 1981. Lipid insertion of cholera toxin after binding to GM1-containing liposomes. *J. Biol. Chem.* 256:11177–11181.

Tran, D., J.L. Carpentier, F. Sawano, P. Gorden, and L. Orci. 1987. Ligands internalized through coated or noncoated invaginations follow a common intracellular pathway. *Proc. Natl. Acad. Sci. USA.* 84:7957–7961.

Walter, P., and G. Blobel. 1983. Preparation of microsomal membranes for cotranslational protein translocation. *Methods Enzymol.* 96:84–93.

Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell.* 83:121–127.

Werner, E.D., J.L. Brodsky, and A.A. McCracken. 1996. Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate. *Proc. Natl. Acad. Sci. USA.* 93:13797–13801.

Wiertz, E.J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T.R. Jones, T.A. Rapoport, and H.L. Ploegh. 1996. Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction. *Nature.* 384:432–438.

Wisnieski, B.J., and J.S. Bramhall. 1981. Photolabelling of cholera toxin subunits during membrane penetration. *Nature.* 289:319–321.

Yang, M., S. Mura, J.S. Bonifacino, and A.M. Weissman. 1998. Novel aspects of degradation of T cell receptor subunits from the endoplasmic reticulum (ER) in T cells: importance of oligosaccharide processing, ubiquitination, and proteasome-dependent removal from ER membranes. *J. Exp. Med.* 187: 835–846.

Yoshida, T., C.C. Chen, M.S. Zhang, and H.C. Wu. 1991. Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and Pseudomonas toxin. *Exp. Cell Res.* 192:389–395.