BiP-dependent export of cholera toxin from endoplasmic reticulum-derived microsomes

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Abstract Cholera toxin (CT) is transported from the cell surface to the endoplasmic reticulum (ER) from where it is translocated to the cytosol in a process depending on ATP and luminal ER proteins. To test whether the molecular chaperone BiP (heavy chain binding protein), which is an ER-luminal ATPase, was one of the required proteins the export of CT was analyzed using ER-derived CT-loaded microsomes. The resubstitution of extracted export-incompetent microsomes with purified BiP was sufficient to restore the export of CT. As BiP protected CT from aggregation it is proposed that BiP maintains CT in a soluble, export-competent state.

Key words: Chaperone; Cholera toxin; Endoplasmic reticulum; Translocation

1. Introduction

Several bacterial and plant toxins act on cytosolic target proteins of the intoxicated cell. To enter the cytosol some of these toxins such as cholera toxin (CT) and ricin travel in a retrograde manner through the secretory pathway to the endoplasmic reticulum (ER) where the actual membrane translocation into the cytosol takes place [1]. Ricin has been found bound to the Sec61p complex during the intoxication process [2] and its translocation across the ER membrane depended on the Sec61p complex in a yeast model system [3] suggesting that this complex mediates the translocation of the toxin. The Sec61p complex forms a protein-conducting pore in the ER membrane and mediates the translocation of secretory proteins into the ER [4]. In ER-derived microsomes the export of subunit A1 of CT (CTA1), which is the subunit actually transferred to the cytosol of the intoxicated cell, was inhibited by blocking the Sec61 complexes with import substrates demonstrating that the same Sec61p complexes which mediate protein import are responsible for the export of the toxin [5]. Misfolded secretory and membrane proteins are retained in the ER and degraded by the proteasome which requires their prior export to the cytosol [6]. As this export is Sec61p-dependent it appears that the toxins use the export machinery of the ER-associated degradation pathway to enter the cytosol. In addition to the translocon, several other membrane proteins such as the Hrd/Der proteins or calnexin as well as the soluble ER-resident molecular chaperone BiP (heavy chain binding protein) [7,8] and protein disulfide isomerase (PDI) [9,10] have been shown to be required for the degradation of misfolded proteins. Whereas PDI has been reported to function in the translocation of CT [11,12] the involvement of BiP has as yet not been determined. The involvement of an ER-resident ATPase such as BiP is, however, an open question because the export of CTA1 from microsomes was shown to be ATP-dependent but independent of cytosolic proteins [5].

Here, we show that the export of CT from microsomes which had been depleted of their luminal proteins and, therefore, were export-incompetent was restored by reconstituting the microsomes with purified BiP. As BiP inhibited the aggregation of CT we propose that BiP keeps CT in a soluble, export-competent state.

2. Materials and methods

2.1. Export assay

The in vitro translation of CTA1, the protease protection analysis and the export assay were performed exactly as described [5]. The export reactions were performed in the absence of cytosol.

2.2. Reconstitution of microsomes

Microsomes were extracted and reconstituted with recombinant BiP (BiP_{rec}) (2 mg/ml) or purified CTA (0.2 mg/ml; Calbiochem) exactly as described [5]. Antibodies were anti-BiP antiserum (Santa Cruz), anti-CT antiserum (Sigma), antiPDI monoclonal (StressGen) or anti-Sec61α antiserum [13]. Enhanced chemiluminescence detection was used.

2.3. Preparation of BiP_{rec}

The cDNA encoding the mature BiP without the signal peptide was cloned from Chinese hamster ovary cells by reverse transcription polymerase chain reaction and inserted into the bacterial expression vector pASK-IBA4 (IBA). This vector introduces an N-terminal StrepTag sequence comprising the amino acids ASWSHPQFEKGA. BiP_{rec} was purified on a StrepTactin agarose column (IBA) according to the manufacturer's protocol and concentrated using Centricon 50 columns (Millipore).

2.4. Preparation of luminal ER proteins (ER extract)

An ER extract was prepared as described [5] with the only exceptions being that 0.25% sodium cholate was used instead of *n*-octylglucoside and 25 mM HEPES–KOH, pH 7.4, 250 mM sucrose, 50 mM

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Abbreviations: BiP, heavy chain binding protein; BiP_{rec}, recombinant BiP; CT, cholera toxin; CTA, CT subunit A; CTA1, CT subunit A1; ER, endoplasmic reticulum; PDI, protein disulfide isomerase

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KCl, 2 mM $MgCl_2$ (extract buffer) was used as buffer during the entire preparation. To deplete the extract of BiP it was passed through an ATP-agarose column (Sigma) equilibrated in extract buffer.

2.5. Aggregation assay

CT (40 nM) was incubated for 20 min at 37°C in extract buffer containing, as indicated, 30 μg/ml ER extract, BiP-depleted ER extract, BiP-depleted ER extract resubstituted with 1 μM BiP_{rec} or 1 μM BiP_{rec} alone. Where indicated the reaction contained 5 mM ATP. The reaction was separated into supernatant and pellet by centrifugation (16 000×*g*, 15 min, 4°C).

2.6. Protease resistance assay

CTA was either denatured by boiling for 5 min in 0.5% sodium dodecyl sulfate (SDS) or kept on ice without SDS. The samples were diluted with 5 volumes phosphate-buffered saline containing 1% Triton X-100. 30 μg/ml (final concentration) ER extract or extract buffer was added (final concentration of CTA: 20 nM). Trypsin treatment was performed for 30 min on ice.

3. Results

The export of CTA1 from microsomes requires ATP and luminal ER proteins [5]. As BiP would, in principle, be sufficient to fulfill both requirements, we directly tested its involvement in the export of CTA1. For this purpose microsomes were either depleted of their luminal proteins (extracted microsomes; Fig. 1a, lane 4) or after extraction resubstituted with purified BiP_{rec} (lane 3). After extraction the luminal proteins BiP and PDI were undetectable. BiP_{rec} was almost completely protected from trypsin added to the outside of the microsomes (Fig. 1b, lane 2), whereas it was readily degraded in the presence of detergent (lane 3) verifying that the resubstituted BiP_{rec} was contained inside the microsomes. The import of in vitro translated CTA1 was equally efficient in both the BiP_{rec}-resubstituted (Fig. 1c, lanes 4–6) and the extracted (lanes 7–9) microsomes as demonstrated by the protection of CTA1 from trypsin added to the outside of the microsomes. However, the microsomes differed drastically in their capacity to export CTA1. Whereas the extracted microsomes (Fig. 2, upper panel, lanes 13–18) were deficient in the export of CTA1 the BiP_{rec}-resubstituted microsomes (lanes 7–12) exported CTA1 almost as efficiently as mock-treated microsomes (lanes 1–6). The ER-resident protein Sec61α was not released into the supernatant (Fig. 2, lower panel). The export of CTA1 was ATP-dependent. It should be mentioned that the export of CTA1 is independent of cytosolic factors such as the Hsp70 chaperones [5], arguing against the possibility that the small fraction of BiP_{rec} which might have been adsorbed to the outside of the microsomes had a function in the export of CTA1.

In a second approach extracted microsomes were resubstituted with CTA purified from *Vibrio cholerae* (Fig. 3a, lanes 3–4) and, where indicated, with BiP_{rec} (lane 4). CTA comprises the translocated CTA1 moiety and the CTA2 chain which remains in the ER. These CTA-resubstituted microsomes faithfully recapitulate the export of CTA1 in that it is dependent on ATP, the Sec61p complex and luminal ER proteins [5]. As this system does not depend on the co-translational import of newly synthesized CTA1, indirect import- or maturation-dependent effects on the export of CTA1 can be excluded. Also in this system, the extracted microsomes (Fig. 3b, upper panel, lanes 1–6) had lost the ability to export CTA1 whereas the BiP_{rec}-resubstituted microsomes (lanes 7–12) efficiently supported its export. Sec61α was not released into the supernatant (Fig. 3b, lower panel). The CTA1 signal seen in the supernatant of the extracted microsomes did not increase during the chase and thus did not represent export.

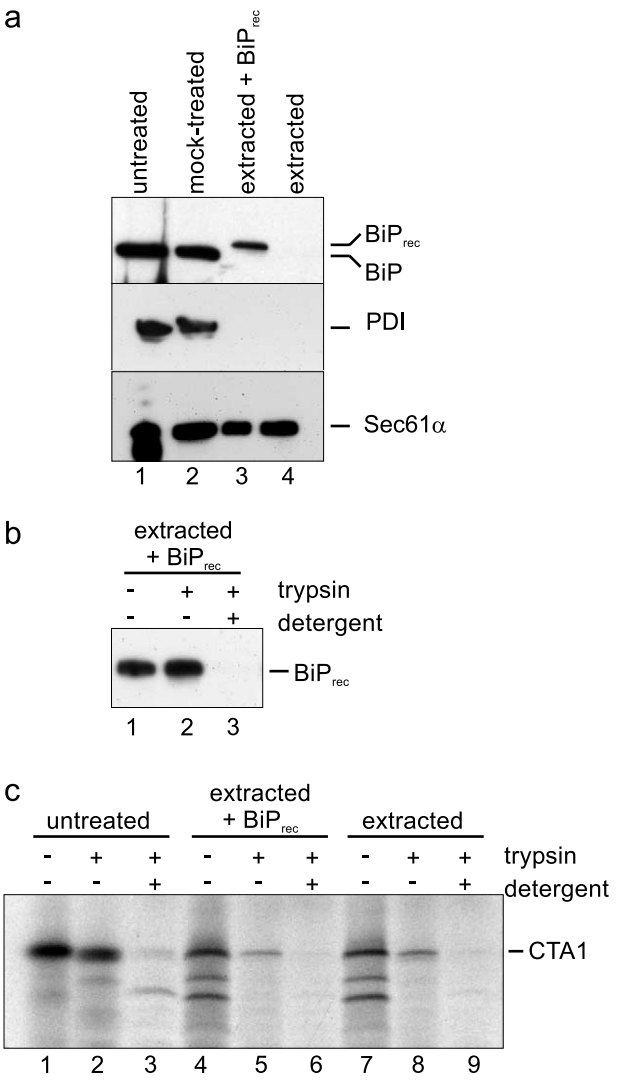

Fig. 1. CTA1 is imported into reconstituted microsomes. a: Microsomes were left untreated (lane 1), depleted of their luminal proteins by detergent extraction (extracted, lane 4) or after extraction resubstituted with recombinant BiP (extracted+BiP_{rec}, lane 3). Mock-treated microsomes (lane 2) went through the entire extraction procedure but the detergent was omitted. BiP, PDI and Sec61α were visualized by immunoblot. b: BiP_{rec}-resubstituted microsomes were treated with 100 μg/ml trypsin in the absence or presence of detergent (50 mM *n*-octylglucoside). BiP_{rec} was visualized by immunoblot. c: Untreated (lanes 1–3), BiP_{rec}-resubstituted (lanes 4–6) or extracted (lanes 7–9) microsomes were loaded with CTA1 by in vitro translation. After re-isolation the microsomes were treated with trypsin or trypsin and detergent (0.5% Triton X-100). CTA1 was visualized by autoradiography.

In yeast BiP has been shown to prevent the aggregation of misfolded secretory proteins [14]. As CTA has a tendency to aggregate the ability of BiP to prevent the aggregation of CTA was tested. For this purpose an ER extract was depleted of BiP (Fig. 4a). Incubation at 37°C of purified CTA in buffer resulted in its quantitative aggregation (Fig. 4b, upper panel, lanes 1–2). The aggregation was largely prevented by the addition of an ER extract (lanes 3–4) but not by a BiP-depleted ER extract (lanes 5–6). BiP_{rec} alone (lanes 9–10) inhibited the

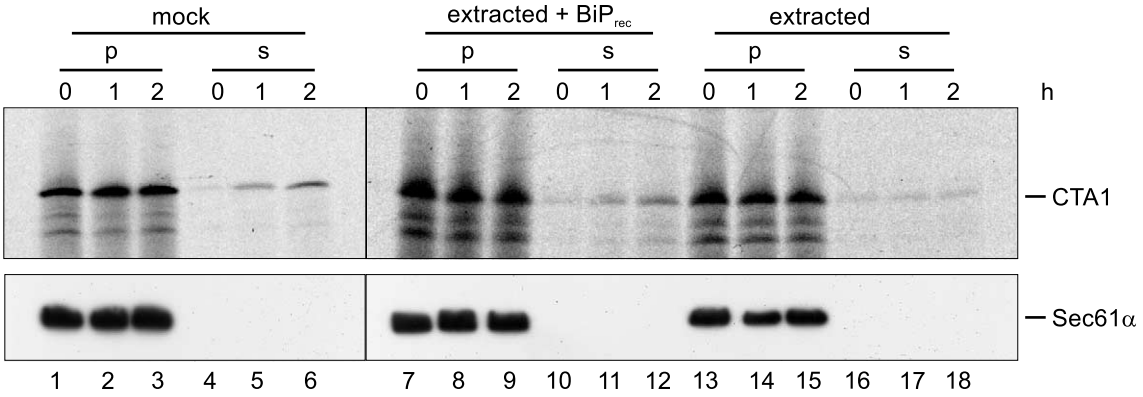

Fig. 2. BiP corrects the export deficiency of extracted microsomes. Mock-treated (lanes 1–6), BiP-resubstituted (lanes 7–12) or extracted (lanes 13–18) microsomes loaded with CTA1 by in vitro translation were incubated in the presence of ATP for the indicated time. At the end, the reaction was separated by centrifugation into a pellet (p) and a supernatant (s). CTA1 was visualized by autoradiography (upper panel) and Sec61α by Western blot (lower panel). Export is defined as the time-dependent increase of CTA1 found in the supernatant.

aggregation of CTA and this inhibition was further enhanced when a substoichiometric amount of a BiP-depleted extract was added together with BiP$_{rec}$ (lanes 7–8). In the presence of ATP the release of substrates from BiP is enhanced [15]. Accordingly, a larger fraction of aggregating CTA was found in the presence of ATP than in its absence and this ATP dependence was seen only in the presence of BiP (Fig. 4b, lower panel). The addition of an ER extract or BiP$_{rec}$ after CTA had been aggregated by incubation in buffer did not solubilize the aggregates (data not shown), indicating that BiP has the ability to prevent the aggregation of CTA but not to solubilize preformed aggregates. To exclude the possibility that CTA aggregated in the absence of BiP because it was denatured its susceptibility to proteolytic degradation was determined. Whereas CTA was already degraded at low concentrations of trypsin after denaturation with SDS (Fig. 4c, upper panel, lanes 1–6) its degradation required much higher concentrations of trypsin in the absence of SDS (lanes 7–12) indicating that it was folded into a compact conformation. CTA incubated with ER extract in the presence of ATP showed similar protease resistance as purified CTA in buffer (Fig. 4c, lower panel) indicating that BiP binding and release did not result in unfolding of CTA. Thus, the aggregation of CTA1 in buffer as well as in the ER extract appears to be due to the local exposure of hydrophobic patches and not to major unfolding of the protein.

4. Discussion

It is assumed that the retro-translocation through the Sec61p channel requires the unfolding of the transport substrate [16]. Protein unfolding could be supported by the ATP-dependent binding and release cycle of BiP. Our results show that the interaction with BiP does not change the protease sensitivity of CTA1 arguing against the assumption that BiP unfolds CTA1. Our observation is in agreement with the major CTA unfolding activity in ER extract being attributed to PDI [11].

In yeast BiP is required for the degradation of carboxypeptidase Y [8] and pro-α factor [7]. Although additional functions cannot be excluded its major function in the degradation of these proteins appears to be to prevent their aggregation [14]. Here, we show that BiP fulfills a similar function during the export of CTA1 from mammalian microsomes suggesting that CTA1 shares the exposure of hydrophobic regions with misfolded proteins. Aggregation results from the exposure of hydrophobic regions which is thought to represent a signal for the ER quality control mechanism to sense a protein as being misfolded [17]. As protease resistance analysis showed that

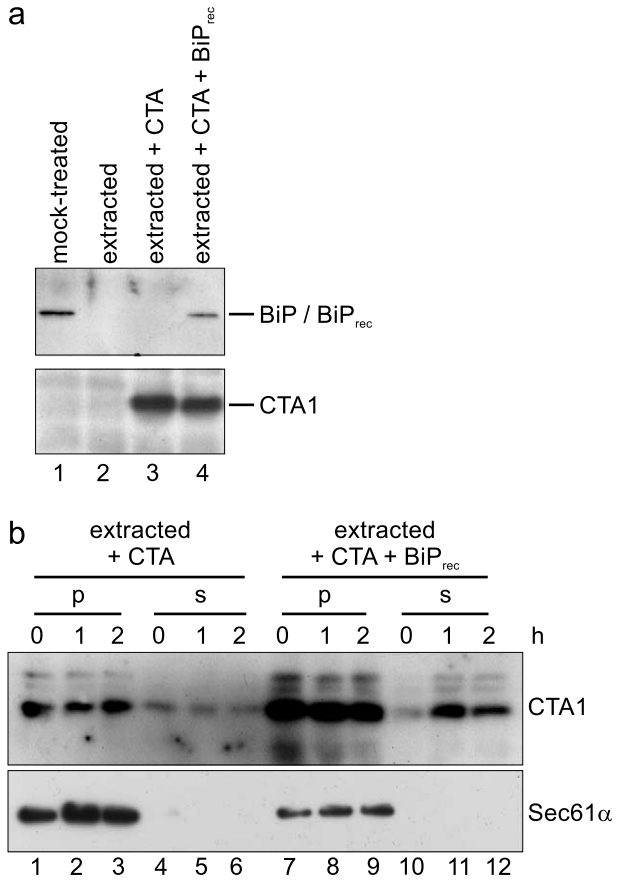

Fig. 3. BiP function is independent of import and folding of CTA1. a: Microsomes were mock-treated (lane 1), extracted (lane 2) or resubstituted with CTA purified from *V. cholerae* (lane 3) or with CTA and BiP$_{rec}$ (lane 4). Immunoblots of BiP (upper panel) and CTA1 (lower panel) are shown. b: Extracted microsomes were loaded with CTA (lanes 1–6) or with CTA and BiP$_{rec}$ (lanes 7–12) and incubated in the presence of ATP for the indicated time. At the end, the reaction was separated by centrifugation into a pellet (p) and a supernatant (s). CTA1 (upper panel) and Sec61α (lower panel) were visualized by Western blot.

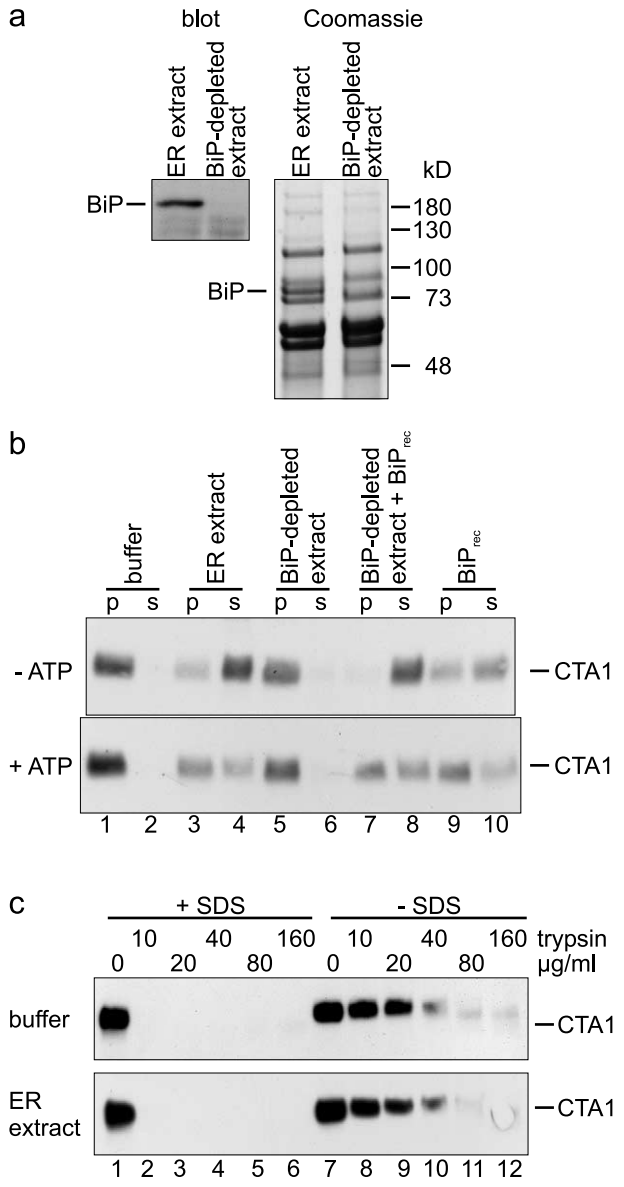

Fig. 4. BiP protects CTA from aggregation. a: Immunodetection of BiP (left) and Coomassie staining (right) of an ER extract and a BiP-depleted ER extract. b: Purified CTA was incubated at 37°C for 20 min in buffer alone (lanes 1–2) or in buffer containing ER extract (lanes 3–4), BiP-depleted ER extract (lanes 5–6), BiP-depleted ER extract complemented with BiP_{rec} (lanes 7–8) or BiP_{rec} without ER extract (lanes 9–10). At the end, the reaction was separated by centrifugation into a pellet (p) containing the aggregated CTA and a soluble fraction (s). CTA1 was visualized by immunoblot. c: Purified CTA was either denatured by boiling in SDS (lanes 1–6) or left untreated (lanes 7–12) and diluted in buffer (upper panel) or in buffer containing ER extract and ATP (lower panel). The samples were incubated for 30 min on ice with the indicated concentrations of trypsin. Immunoblots of CTA1 are shown.

CTA1 was folded into a compact conformation the exposure of the hydrophobic regions appears to occur only for short instances or to be locally restricted. Both temporally [18] and locally restricted [19] unfolding have been shown to be sufficient for a protein being recognized as misfolded. Thus, the limited exposure of hydrophobic regions may be the signal targeting CTA1 to the site of retro-translocation, probably the translocon. It is, however, unlikely that BiP is the targeting factor because CTA1 was released from and rebound to BiP in the absence of microsomal membranes. As the addition of an ER extract enhanced the binding of purified BiP to CTA1, regulatory proteins such as J-domain proteins are likely to modulate these cycles of binding and release. Without excluding possible additional functions we propose a dual function for BiP during retro-translocation: first it keeps the proteins in a soluble and thus export-competent state as has been shown here for CTA1 and by others for carboxypeptidase Y and pro-α factor [14]. Second, by binding to and thus hiding exposed hydrophobic regions BiP might act as a timer. Only after the ATP-dependent release from BiP is the protein accessible to factors targeting the protein for retro-translocation. This function may be important to prevent the premature degradation of proteins which are still in the folding or assembly process and, therefore, still expose hydrophobic regions on their surface. Although this second function is speculative it gains indirect support from two observations. First, the half-life of a non-secreted immunoglobulin light chain correlated with its release from BiP [20]. Second, exposed hydrophobic regions of proinsulin assembly intermediates were permanently shielded by BiP whereas those of a misfolded and degraded proinsulin were not [21].

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