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Ricin A chain utilises the endoplasmic reticulum-associated protein degradation pathway to enter the cytosol of yeast

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Abstract Cytotoxic proteins such as ricin A chain (RTA) have target substrates in the cytosol and therefore have to reach this cellular compartment in order to act. RTA is thought to translocate into the cytosol from the lumen of the endoplasmic reticulum (ER), although how it traverses the ER membrane has not been established. Using yeast mutants defective in various aspects of the ER-associated protein degradation (ERAD) pathway, we show that RTA introduced into the yeast ER subverts this pathway to enter the cytosol via the Sec61p translocon. A significant proportion of the exported RTA avoided proteasomal degradation. These data are consistent with the contention that the RTA component from ricin endocytosed by mammalian cells may likewise exploit ERAD to translocate into the cytosol.

Key words: Ricin A chain; Endoplasmic reticulum; Proteasomal degradation; Sec61p

1. Introduction

The plant protein ricin is a member of the A-B protein toxin family in which a catalytically active A polypeptide, in the case of ricin a ribosomal RNA N-glycosidase [1], is joined to a cell-binding B polypeptide. After entering susceptible mammalian cells by endocytosis, that proportion of the endocytosed ricin responsible for intoxicating the cell is believed to undergo retrograde transport to reach the endoplasmic reticulum (ER) lumen [2,3], from where ricin A chain (RTA) translocation into the cytosol is proposed to occur [4]. Several other bacterial toxins are also believed to undergo retrograde transport to reach the ER as a prerequisite for entering the cytosol [3,5].

The mechanism by which toxic proteins translocate to the cytosol from the ER lumen is not clear. Studies using *Saccharomyces cerevisiae* and mammalian systems have demonstrated that glycopeptides can be released from the ER in an ATP- and cytosol-dependent fashion [6,7]. It has also become clear that proteins failing to fold or oligomerise correctly are not dispatched into ER-derived transport vesicles but are instead targeted for degradation by a non-lysosomal process [8]. Some aspects of this ER-associated protein degradation (ERAD) pathway have now been elucidated [9,10]. It appears that aberrant proteins are retained in the ER and exported to the cytosol [11–14], with most ERAD substrates then being degraded by the cytosolic ubiquitin/proteasome system [9,12,14,15–17]. It has been proposed that toxins such as ricin A chain might also gain access to the cytosol from the ER by masquerading as ERAD substrates [18,19]. In this case there are crucial differences, however: ricin A chain is a native rather than an aberrant protein, and at least a proportion of the exported protein must avoid the ultimate fate of endogenous ERAD substrates, namely proteolytic degradation.

Studying the translocation and fate of RTA in mammalian cells is difficult due to the extreme potency of this toxin and the very low levels that reach the ER. Here we have used *S. cerevisiae* as a model system to examine whether RTA can be exported from the ER by ERAD. While ricin readily intoxicates mammalian cells, externally added toxin does not intoxicate yeast cells, since, lacking galactosyl transferase [20], they do not have galactosylated surface components able to bind ricin B chain (RTB) [21]. To overcome this limitation, we have introduced RTA directly into the yeast ER in vivo. The ERAD systems of mammalian cells and yeast appear to be mechanistically similar, with the export step utilising the Sec61p translocon [16,22,23]. If RTA does use ERAD to translocate across the mammalian ER membrane, it seems likely that the yeast ERAD system would also be capable of exporting RTA, a contention confirmed by the data presented here.

2. Materials and methods

2.1. Plasmid construction and yeast strains

PCR was used to amplify the Kar2 signal sequence from a *S. cerevisiae* genomic preparation. Recombinant PCR was then used to fuse this signal with the various ricin A chain sequences (wild-type/E177D/Δ). *Eco*RI/*Xho*I preRTA fragments were then sub-cloned into the p31GAPFL-IT vector behind the GAPDH promoter creating expression cassettes. These cassettes were then sub-cloned as either *Sal*I/*Pst*I or *Sal*I/*Hin*dIII fragments into the yeast expression vectors pRS315 (LEU2) or pRS316 (URA3) [24], respectively. Yeast strains were transformed with the RTA expression plasmids using the lithium acetate method, selecting for transformants on minimal medium with the appropriate supplements. Yeasts mutant strains (and their isogenic wild-types) transformed with the RTA expression plasmids are described elsewhere: Δ*der1* [25]; *cim3-1*, *cim5-1* [26]; Δ*ubc6*, Δ*ubc7*, Δ*ubc6/7* [27,28]; *sec61-32*, *sec61-41* [22].

2.2. In vitro transcription and translation

For in vitro experiments, the *Eco*RI/*Xho*I RTA fragments were subcloned into the phagemid pGEM11Zf(+) (Promega, Madison, WI, USA). T7 polymerase (Gibco BRL, Paisley, UK) was used to create

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Abbreviations: RTA, ricin toxin A chain; RTB, ricin toxin B chain; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated protein degradation

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RNA transcripts which were then translated in a wheatgerm system containing [^{35}S]methionine (Amersham International, Amersham, UK) at 28°C for 1 h. For protease digestion experiments, aliquots of the in vitro translated ricin A chain products were incubated with equal volumes of thermolysin (Boehringer, Mannheim, Germany), at various concentrations, on ice for 45 min, followed by SDS-PAGE analysis.

2.3. Pulse chase analysis

The fate of in vivo expressed ricin A chain was followed in pulse/chase experiments in a similar manner to that described earlier [17]. Briefly, cells were subcultured in minimal medium with appropriate additions until they were in log phase. All incubations were carried out at 30°C, except for the experiments using the cold sensitive Sec61 mutants, in which case cells were incubated at 24°C (the restrictive temperature for ER export). 25 OD_{600} units of cells were taken and resuspended in minimal medium lacking methionine and pulsed for 10 min with 25 μCi/OD_{600} of [^{35}S]methionine/cysteine (ProMix; Amersham International, Amersham, UK). An equal volume of chase medium (minimal medium containing 6 mg/ml methionine) was then added and aliquots were removed for analysis at the appropriate chase times. Cell lysates were prepared as described [17] and analysed by 12.5% SDS-PAGE. Samples for endoH treatment were resuspended in 10 mM Tris-HCl, pH 6.8, 1% SDS and then incubated with 10 mU of endoH (ICN Biomedicals, Costa Mesa, CA, USA) and sodium citrate pH 5.5 at 37°C for 24 h prior to SDS-PAGE analysis. Samples were quantified on a Molecular Dynamics phosphorimager (Sunnyvale, CA, USA).

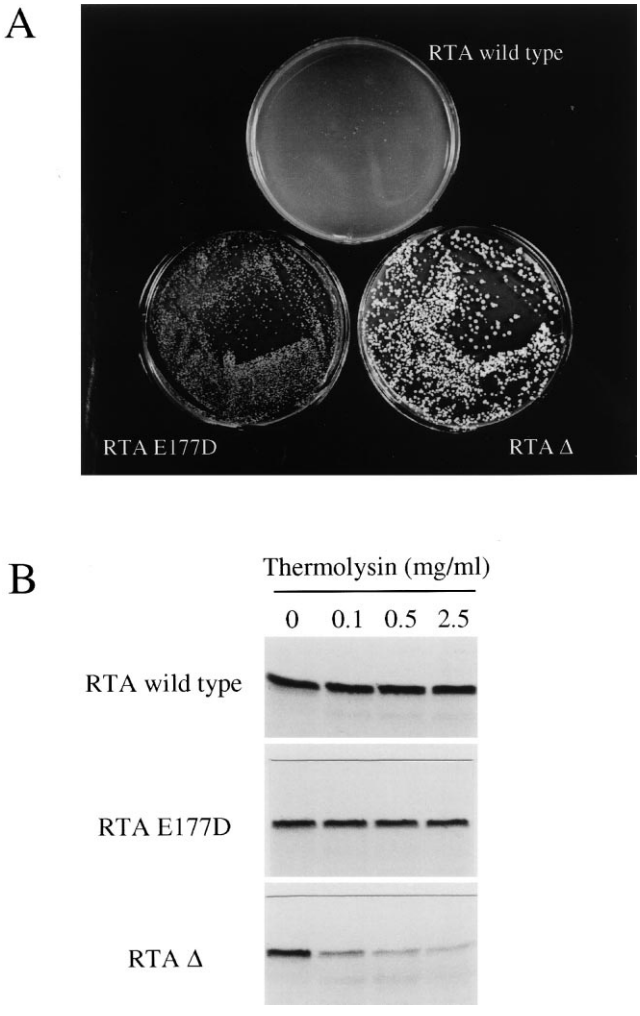

Fig. 1. *S. cerevisiae* cells can tolerate expression of correctly folded ricin A chain E177D, but not wild-type. A: Wild-type cells were transformed with expression plasmids encoding Kar2-RTA (wild-type/E177D/Δ) and incubated on minimal medium plates at 30°C for 3 days. B: RTA wild-type and E177D show identical protease-resistance to thermolysin, whereas as RTAΔ is efficiently degraded. RTA variants were translated in vitro, then incubated with thermolysin at the concentrations indicated. Labelled RTA remaining after this time was analysed by 12.5% SDS-PAGE and autoradiography.

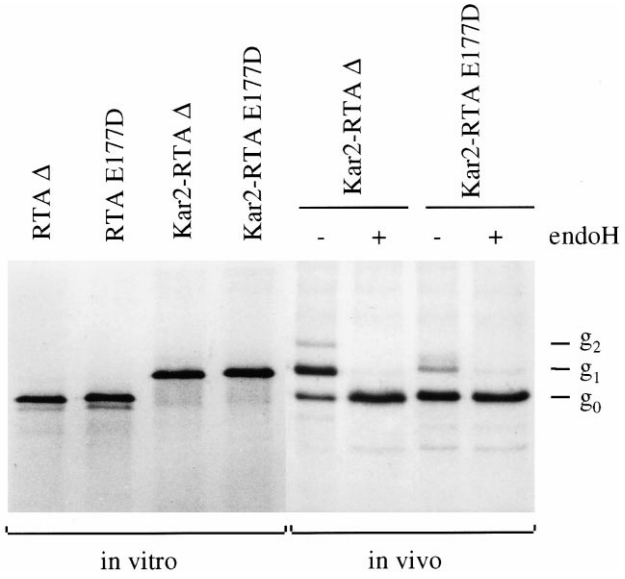

Fig. 2. Expressed Kar2-RTA is efficiently signal sequence cleaved and glycosylated in yeast cells. In vitro wheatgerm translation reactions were used to define the sizes of RTA with and without the Kar2 signal sequence (lanes 1–4). Yeast cells expressing Kar2-RTAΔ or Kar2-RTA E177D were pulse labelled with [^{35}S]methionine/cysteine for 10 min and homogenised. RTA was recovered by immunoprecipitation, and either not treated (−) or treated (+) with endoH (lanes 5–8). Products were analysed by SDS-PAGE and visualised by autoradiography.

3. Results

3.1. Expression of RTA in yeast

Yeast cells were transformed with expression vectors encoding RTA preceded by the yeast Kar2 signal sequence. Although this signal peptide effectively delivers RTA into the ER lumen (see below), cells expressing Kar2-RTA(wild-type) were killed (Fig. 1A), possibly because ER-segregated RTA was subsequently translocated to the cytosol. To overcome this toxicity, we used an RTA mutant in which the active site Glu-177 residue had been conservatively changed to Asp. RTA E177D retained catalytic activity, indicating a native conformation, but had a 50-fold reduction in k_{cat} [29] that permitted the survival of yeast cells expressing this protein (Fig. 1A). We also produced a misfolded RTA mutant, known as RTAΔ, possessing an active site deletion which is completely devoid of catalytic activity. The native RTA conformation is very resistant to proteolytic digestion, but incorrectly folded conformations are not [30]. As shown in Fig. 1B, both wild-type RTA and RTA E177D were resistant to thermolysin digestion under conditions that resulted in the degradation of RTAΔ, further supporting the contention that RTA E177D folds into the native conformation.

The Kar2 signal peptide quantitatively directed RTA into the ER lumen both in vitro and in vivo (Fig. 2). In vivo, three immunoreactive bands were seen representing non-glycosylated, signal-cleaved RTA (g_0) and RTA glycosylated at one

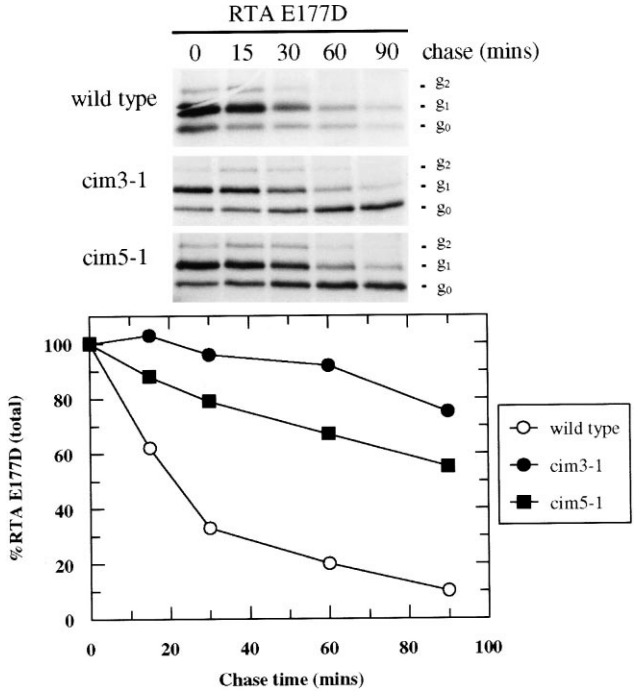

Fig. 3. Effects of mutations in the proteasome on the rates of degradation of RTA E177D. Wild-type yeast or yeast cells harbouring either the *cim3-1* or *cim5-1* mutations were transformed with a plasmid encoding Kar2-RTA E177D, pulse labelled with [^{35}S]methionine/cysteine for 10 min and chased for the times indicated. RTA products were recovered from cell homogenates by immunoprecipitation, analysed by SDS-PAGE and visualised by fluorography (upper panels) and quantified using a phosphorimager (lower panels). Each point for the quantitation represented the sum of all glycosylated RTA forms and the non-glycosylated form.

(g_1) or both (g_2) of its two N-glycosylation sites. Protease protection experiments also indicated efficient segregation into the ER lumen in that RTAE177D within microsomes isolated from cells pulse-labelled with [^{35}S]methionine/cysteine were completely resistant to proteinase K digestion but were fully digested when the protease was added in the presence of detergent (data not shown). It should be noted that active preparations of proteinase K can degrade native RTA; the differential degradation shown in Fig. 1B was seen with thermolysin.

3.2. RTA is exported from the ER by the ERAD pathway

We pulse-labelled wild-type yeast cells and cells defective in the Cim3p or Cim5p subunits of the 19S cap of the 26S proteasome complex, each of which was expressing RTA E177D, with [^{35}S]methionine/cysteine for 10 min followed by a chase with unlabelled amino acids for 90 min. As shown in Fig. 3, RTA E177D degradation was significantly retarded in *cim3-1* and *cim5-1* cells. The rate of RTA E177D degradation was not reduced in yeast cells devoid of the ER protein Der1p (data not shown), in contrast to CPY* whose degradation is abolished in such cells [25].

The rate of degradation of RTA E177D in yeast mutants defective in ubiquitination was not significantly different from the rate in wild-type cells (data not shown). The deletion of the *UBC6* gene, which encodes a ubiquitin-conjugating enzyme Ubc6p present in the ER membrane with its catalytic domain exposed to the cytosol [27], the deletion of *UBC7*, which encodes the soluble yeast ubiquitin-conjugating enzyme Ubc7p [28], or the simultaneous deletion of both *UBC6* and *UBC7* did not affect the rate of RTA degradation compared to wild-type cells, even though the mutant phenotype was confirmed by the reduced rate of degradation of the carboxypeptidase yscY mutant CPY* in these strains [15] (data not shown).

RTA was exported from the ER via the Sec61p translocon. Pulse-labelled RTA E177D, which was rapidly exported from the ER and degraded in wild-type yeast, was stabilised in both *sec61-32* and *sec61-41* cells (Fig. 4). At the permissive temperature for protein import into the ER, these mutants are defective in ER export [22].

3.3. Some of the RTA exported to the cytosol escapes proteasomal degradation

If native RTA exploits ERAD for export from the ER to the cytosol, then clearly some of the exported protein must avoid immediate proteasomal degradation in order to depurinate sufficient ribosomes to cause cell death. To determine if a proportion of the RTA escapes proteasomal degradation, we pulse-labelled wild-type cells that were expressing RTA E177D as before, but continued the subsequent chase for an extended period of 4 h. As shown in Fig. 5, while most of the RTA was degraded during the first hour of the chase, around 20% was not degraded but appeared to be completely stable. Cell fractionation showed that this stable RTA was in the soluble, rather than the microsomal fraction, indicating that it was cytosolic (data not shown). This stabilisation appeared to be significant, since in pulse-chase experiments similar to those used in the present study, misfolded CPY* was completely degraded after 60 min of chase [15]. The fact that yeast

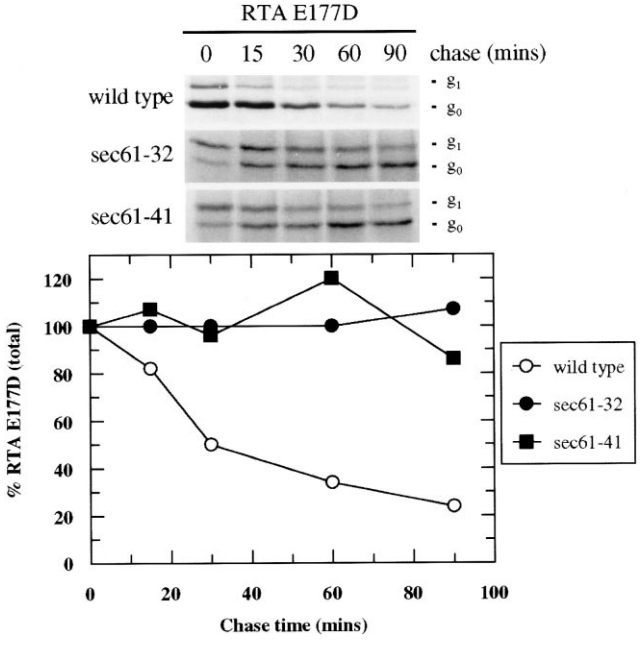

Fig. 4. The rate of RTA E177D degradation was markedly reduced in yeast mutants defective in protein export via the Sec61p translocon. Wild-type yeast or yeast cells carrying the *sec61-32* or *sec61-41* mutations were transformed with a plasmid encoding Kar2-RTA E177D, incubated at 24°C and subjected to pulse-chase analysis as described in the legend to Fig. 3. The upper panels show the autoradiograms and the lower panels the quantitation of total RTA at each chase point.

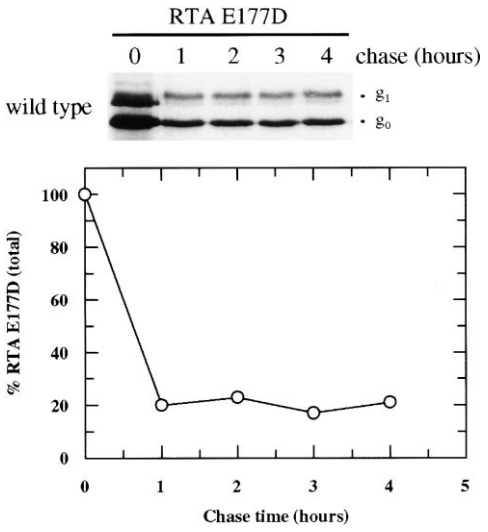

Fig. 5. A proportion of RTA E177D is resistant to proteolytic degradation. Wild-type yeast cells were transformed with a plasmid encoding Kar2-RTA E177D and subjected to pulse-chase analysis as described in the legend to Fig. 3, except the chase was performed for 4 h. The upper panel shows the autoradiogram and the lower panel the quantitation of total RTA E177D at each chase point.

cells expressing RTA E177D were able to grow while producing catalytically active toxin indicates that the rate of ribosome inactivation was not limiting.

4. Discussion

The data presented here show that an active form of RTA (RTA E177D), which appears to assume the native conformation, acts as a substrate for the ERAD pathway when introduced into the ER lumen of yeast. This supports the notion that RTA reaching the ER lumen of mammalian cells after endocytic uptake and retrograde transport [2] could also subvert this pathway to gain access to the cytosol.

In yeast, Der1p, which has been implicated in the export of misfolded yeast proteins [25], does not appear to play a role in the export of RTA. Furthermore, RTA is not a substrate for ubiquitination as evidenced by degradation within cells defective in the ubiquitin conjugating enzymes Ubc6p and Ubc7p, possibly because of its paucity of lysines [19]. However, proteasomal degradation is the fate of the majority of exported RTA (Fig. 4). Ubiquitin-independent, proteasomal-dependent degradation can occur in yeast [12] and mammalian cells [31]. Where export is seen, there is a noticeable increase in the level of de-glycosylated RTA with time and a concomitant disappearance of glycosylated species in the various mutants. This is suggestive of a cytosolic PNGase activity which would be expected in such stressed cells [32]. In mammalian cells, translocation of RTA to the cytosol is a costly process. Treating such cells with the proteasomal inhibitor lactacystin increases their sensitivity to ricin three- to four-fold (D.C. Smith, L.M.R. and J.M.L., unpublished) implying that the bulk of the RTA that enters the cytosol is degraded. RTA refolds in the cytosol in lethal quantities, however, since cell death ensues even in the face of high levels of proteasomal degradation

Here we have introduced RTA into the ER lumen in the absence of RTB, although RTA reaching this compartment during endocytic uptake would be part of the ricin holotoxin. RTB is believed to function in cell entry and intracellular transport but does not appear to play a role in the membrane translocation step. Many RTA-containing immunotoxins which lack RTB are just as potently cytotoxic as the ricin holotoxin with IC_{50} values $\sim 10^{-12}$ M [33]. Furthermore, free RTA taken into cells by fluid phase uptake and transport to the ER via the Golgi has a toxic effect [34], something that is, in effect, mimicked here by the introduction of nascent RTA to the yeast ER lumen. Breaking the interchain disulfide bond linking RTA and RTB is essential for cytotoxicity [35], and this event, by analogy to cholera toxin [36,37], is thought to occur with the ER lumen. Releasing RTA from RTB would expose a hydrophobic region at the C-terminus of RTA, possibly triggering interaction with membranes, chaperones or Sec61p translocons directly. Although the factors and mechanisms involved are not yet known, it seems very likely that reduced, native RTA within the ER lumen is perceived as an ERAD substrate. As such it is exported as a glycoprotein to the cytosol, as occurs here from the yeast ER.

If proteasomal degradation is the fate of most RTA, how does a proportion avoid proteasomal degradation in order to kill the cell? As noted earlier, while native RTA is extremely resistant to proteolytic degradation, misfolded or partially unfolded RTA is protease-sensitive. In order to become translocationally-competent, RTA presumably has to partially unfold. Immediately after membrane translocation, partially unfolded RTA would be susceptible to proteasomal degradation, but if a proportion of the translocated protein regained its native conformation, it could become resistant to proteasomal action.

Regardless of the mechanisms employed, our data clearly show that RTA can translocate from the yeast ER by acting as a substrate for the ERAD pathway. Thus protein toxins, in addition to viruses [13], may be able to use the ERAD pathway to their advantage. By capitalising on the normal eukaryotic cellular functions of endocytosis, transport from endosomes to the *trans*-Golgi network [2], retrograde vesicular transport from the Golgi complex to the ER [2], and the ERAD pathway, these toxins are able to traverse the entire secretory pathway in a retrograde fashion and deliver a biologically-active protein from outside of the cell to the cytosol.

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