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Efficient export of human growth hormone, interferon α2b and antibody fragments to the periplasm by the *Escherichia coli* Tat pathway in the absence of prior disulfide bond formation

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ABSTRACT

Numerous therapeutic proteins are expressed in *Escherichia coli* and targeted to the periplasm in order to facilitate purification and enable disulfide bond formation. Export is normally achieved by the Sec pathway, which transports proteins through the plasma membrane in a reduced, unfolded state. The Tat pathway is a promising alternative means of export, because it preferentially exports correctly folded proteins; however, the reducing cytoplasm of standard strains has been predicted to preclude export by Tat of proteins that contain disulfide bonds in the native state because, in the reduced state, they are sensed as misfolded and rejected. Here, we have tested a series of disulfide-bond containing biopharmaceuticals for export by the Tat pathway in CyDisCo strains that do enable disulfide bond formation in the cytoplasm. We show that interferon α2b, human growth hormone (hGH) and two antibody fragments are exported with high efficiency; surprisingly, however, they are efficiently exported even in the absence of cytoplasmic disulfide formation. The exported proteins acquire disulfide bonds in the periplasm, indicating that the normal disulfide oxidation machinery is able to act on the proteins. Tat-dependent export of hGH proceeds even when the disulfide bonds are removed by substitution of the Cys residues involved, suggesting that these substrates adopt tertiary structures that are accepted as fully-folded by the Tat machinery.

1. Introduction

Escherichia coli is a popular host for the production of recombinant proteins, underpinning the production of over a third of currently-licensed therapeutic proteins [28]. There are several strategies for production of these biopharmaceuticals in *E. coli*, including expression of soluble proteins in the cytoplasm, expression as insoluble inclusion bodies or export to the periplasm followed by selective rupturing of the outer membrane to release the protein [22]. The latter is a favoured approach for many protein products, because it offers major advantages in downstream processing, including a reduction in the release of contaminant proteins and proteases, a lack of DNA release and less debris micronisation, hence better clarification performance (reviewed in [2, 11]). In addition, the periplasm is an oxidising environment, which is essential for the production of disulfide bond-containing proteins. In wild type *E. coli* host strains, these cannot form in the cytoplasm.

Export of biopharmaceuticals to the periplasm is normally achieved by attachment of an N-terminal 'Sec'-type signal peptide which directs export via the general secretory (Sec) pathway (reviewed in [9]). This system transports protein substrates through a membrane-bound translocase in an unfolded form, after which the protein folds in the periplasm and any disulfide bonds are formed at this point. However, a high proportion of heterologous proteins are Sec-incompatible because they fold too rapidly, or too tightly, for the Sec pathway to handle effectively. In this context, the twin-arginine translocation (Tat) system offers a potentially important alternative to the Sec pathway. It operates in parallel with the Sec pathway in most bacteria but uses a completely different translocation mechanism. As with Sec substrates, Tat substrates are synthesised with N-terminal signal peptides, but these contain specific determinants including the presence of a highly conserved twin-arginine motif. The Tat pathway has two unique properties that have major implications for its exploitation.

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First, it transports fully-folded proteins (for reviews see [19,26]) and therefore has real potential for the export of proteins with folding properties that preclude Sec-dependent export. An example is green fluorescent protein (GFP), a tightly-folding protein that cannot normally be exported by Sec, but which is very efficiently exported by Tat [27]. Moreover, the Tat pathway can support levels of protein export that are comparable to those obtained in Sec-dependent industrial production systems; fed-batch fermentation studies showed that periplasmic GFP levels exceeded 1 g protein per litre culture [16].

A second Tat trait is also highly relevant for biotechnological exploitation purposes: not only is Tat capable of exporting fully folded proteins, there is widespread evidence that it preferentially exports correctly folded proteins — even heterologous proteins. Indeed, studies on mutated versions of several substrates have shown an all-or-nothing effect, in that misfolded proteins are essentially 100% rejected (for example [10,17]; reviewed in [26]). The underlying quality control mechanism is poorly understood, but there is evidence that the proofreading ability of the Tat pathway can involve at least two elements: co-factor insertion and substrate assembly mediated by protein chaperones such as DmsD, HybE, NapD and TorD [6,20] or direct interaction with the translocase itself (for review see [21]). These reports showed that misfolded proteins, with even minor changes in structure, could lead to reduced or blocked translocation, although some exceptions have been noted; for example Richter et al. [25] showed that small, unstructured hydrophilic proteins (<30 kDa) could be transported. In general, this proofreading ability is poorly understood, but however it works, the trait is potentially important for biotechnological exploitation of Tat, because it implies that exported products should have high specific activities and minimal heterogeneity.

While this trait has interesting implications for the use of Tat in production platforms, there is evidence that it may cause problems for the export of disulfide-bond requiring proteins. Several proteins which contain disulfide bonds in the native state could not be exported by Tat, because they were found to be misfolded, and hence completely Tat-incompatible, in the absence of cytoplasmic disulfide formation [5,16]. However, some proteins were exported in an *E. coli* mutant strain, *Δgor/ΔtrxB*, that allows formation of the disulfide bonds in the cytoplasm [5]. Similar results were obtained by our group when we expressed disulfide-requiring proteins in 'CyDisCo' (Cytoplasmic Disulfide formation in *E. coli*) strains that promote efficient formation of disulfide bonds in the cytoplasm [16]. The Tat pathway thus clearly identified these constructs as misfolded in wild type strains and rejected them. Hence, Tat-dependent export required prior oxidative folding.

CyDisCo strains express a yeast mitochondrial thiol oxidase, Erv1p and human protein disulfide isomerase (PDI) which confer the ability to catalyse cytoplasmic disulfide bond formation and isomerization and these factors were shown to enable the Tat-dependent export of two disulfide-requiring model proteins, namely PhoA and AppA. These results suggested that it has promise for the export of disulfide-requiring biopharmaceuticals, and in this study we have tested a series of such proteins for export by the Tat pathway. We show that the proteins are all efficiently exported in Erv1p/PDI-expressing cells; a major surprise, however, is the finding that export is equally efficient in wild type cells. In the absence of Erv1p and PDI, the proteins are shown to acquire disulfide bonds in the periplasm, and we speculate that this subset of proteins are exported by Tat in the reduced form because they adopt a near-native structure that enables them to avoid rejection by the Tat pathway's proofreading system.

2. Materials and methods

2.1. Materials

All chemicals, unless specified otherwise, were obtained from Sigma Chemical Co. Ltd. (Poole, Dorset, UK) and were of analytical grade.

2.2. Plasmids & bacterial strains

All constructs were amplified using Phusion high fidelity DNA polymerase (New England Biolabs) to include a 5′ NdeI site and a 3′ BamHI site immediately after a 6-His tag. The product was then digested with NdeI and BamHI (New England Biolabs) and inserted into pYU49 which had been cut similarly [16].

All plasmid purification was performed using the QIAprep spin miniprep kit (Qiagen) and all purification from agarose gels was performed using the gel extraction kit (Qiagen), both according to the manufacturer's instructions. All plasmids generated were fully sequenced (see Table 1 for plasmid names and details). The sequence of the VH domain construct is shown in the Supplementary data section.

2.3. Growth conditions

Following transformation, a single colony was used to inoculate 10 mL of LB media containing 100 μg mL^{-1} ampicillin and grown overnight at 37 °C, 200 rpm. Using the overnight culture, 50 mL of LB in i L flasks containing appropriate antibiotics was inoculated to OD_{600} = 0.05 and grown to approximately OD_{600} = 0.5 at 37 °C, 200 rpm. At OD_{600} = 0.5, cells were induced with 1 mM IPTG and left for 3 h at 25 °C or 30 °C (see main text), 200 rpm. After this time an amount equivalent to 1 mL of OD_{600} = 10 was collected by centrifugation (3000 rpm, 10 min). The periplasmic fraction (P) was prepared by the EDTA/lysozyme/cold osmoshock procedure [22,24]. After removal of the periplasm supernatant the spheroplast pellet was then washed with buffer containing 50 mM Tris–Acetate (pH 8.2), 250 mM sucrose and 10 mM $MgSO_4$ and centrifuged for 5 min at 14,000 rpm, 4 °C. The resulting pellet was then resuspended in 50 mM Tris–Acetate, 2.5 mM EDTA (pH 8.2) and sonicated on ice for 4–6 × 10 s, 8 μm amplitude with 10 s between sonication (Soniprep 150plus, Sanyo Gallenkamp,

Table 1
Strains and constructs used in this study.

Strain/plasmid	Description	Source/reference
DH5α	F_ _80lacZ_M15_(lacZYA-argF) U169 deoR recA1 endA1 hsdR17 (RK_,mk_) gal-phoA supE44 thi-1 gyrA96 relA1	Invitrogen, Carlsbad, CA, USA
W3110	F- mcrA mcrB IN(rrnD-rrnE)1 lambda-	ATCC, Manassas, VA, USA
MC4100, Ara^R	AraR, FaraD139DlacU169 rpsL150 relA1 flB5301 deoC1 ptsF25 rbsR	Bolhuis et al. [4]
ΔTatABCDE, Ara^R	Like MC4100 Ara^R; ΔtatABCDE	Wexler et al. [29], Bolhuis et al. [4]
K12, ΔdsbB	Δ(araD-araB)567 ΔLacZ4787(::rrnB-3) λ⁻ dsbB774::kan rph-1 Δ(rhaD-rhaB) 568 hsdR514	Baba et al. [1]
BL21(DE3)	fhuA2 [lon] ompT gal (λ DE3) [dcm] ΔhsdS λ DE3 = λ sBamHIo ΔEcoRI-B int::(lacI::PlacUV5::T7 gene1) i21 Δnin5	Miroux and Walker [18]
pYU49	pET23 based vector with pTac promoter expressing $TorA_{sp}$ IL-1β scFv, codon-optimised Erv1p and mature codon-optimised hPDI	Matos et al. [16]
pHAK1	As above with mature hGH-6His replacing IL-1β scFv	This study
pHAK2	As above with mature α2b IFN-6His replacing IL-1β scFv	This study
pHAK7	As above with β-galactosidase scFv-6His replacing IL-1β scFv	This study, Martineau et al. [13]
pHAK12	As above with VH domain-6His replacing IL-1β scFv	This study
pHAK14	As pHAK1 but without Erv1p and PDI	This study
pHAK15	As pHAK2 but without Erv1p and PDI	This study
pHAK17	As pHAK7 but without Erv1p and PDI	This study
pHAK23	As pHAK12 but without Erv1p and PDI	This study
pKWK1	As pHAK1 with C53S mutation	This study
pKWK2	As pHAK1 with C189S mutation	This study
pKWK3	As pHAK1 with C53S C189S mutation	This study

Loughborough, UK). The sonicate was then centrifuged for 30 min at 70,000 rpm, 4 °C to collect the insoluble fraction. The supernatant was removed as the cytoplasmic fraction (C) and the pellet resuspended in 50 mM Tris–Acetate, 2.5 mM EDTA (pH 8.2.) to give the membrane/insoluble fraction (M). All cell fractions were stored frozen in aliquots for further experiments as repeated freeze-thawing can influence the results obtained in a protein-dependent manner.

2.4. Detection of proteins by immunoblotting; protein purification

After SDS-PAGE and transfer, PVDF membranes to be immunoblotted with C-terminal His antibodies were blocked with PBS-T containing 5% (w/v) dried skimmed milk powder for at least 1 h. The membranes were washed in PBS-T before incubation with PBS-T containing the primary antibody (Anti-His (C-term), Life Technologies, CA, USA) for 1 h. The membranes were washed before incubation with the secondary antibody (Anti-Mouse IgG (H + L), HRP Conjugate, Promega, WI, USA) for another hour. The membranes were washed and immunoreactive bands were detected using an ECL (enhanced chemiluminescence) kit (BioRad, Herts, UK) according to the manufacturer's instructions. Membranes were developed using a BioRad chemiluminescence imager and corresponding software. hGH was purified by expression of TorA-hGH in 1.2 L wild type cells and purification from the periplasmic extracts, or expression in *tat* mutant cells and purification from the cytoplasmic fraction. The extract was subjected to IMAC chromatography in the presence of 20 mM Tris-HCl, pH 8.6 and the protein was eluted in 50 mM EDTA. The elute was desalted and subjected to Q-Sepharose chromatography in the same buffer, with elution carried out by addition of the same buffer containing 300 mM NaCl.

2.5. Mass spectrometry: in-gel digestion of proteins

Excised, diced protein bands from Coomassie-stained SDS gels were washed, reduced and S-alkylated essentially as described in Fox et al. [8]. A sufficient volume of 2 ng/μL of trypsin (modified sequencing grade, Promega, Southampton, UK) in 25 mM ammonium bicarbonate was added to cover the gel pieces and digestion performed overnight at 20 °C. The digests were then acidified by the addition of a 0.5 volume of 50% acetonitrile with 5% formic acid prior to MS and MS–MS analysis.

2.6. MS, MS–MS and Electrospray MS analysis

The sample (1 μL of the above peptide digest) was placed on the sample target (AnchorChip standard, 800 μm) and dried. Subsequently 0.5 μL of matrix was added and dried. The matrix was α-cyano-4-hydroxy-cinnamic acid (α-CHCA, 0.7 mg mL^{-1} in 85% acetonitrile, 15% H_2O, 0.1% TFA and 1 mM $NH_4H_2PO_4$). For external calibration in the protein mass range, Peptide Calibration Standard I (Bruker) standards were used. MALDI TOF MS and MALDI TOF–TOF MS–MS analyses were performed (in the positive ion mode) using a Bruker UltrafleXtreme. The spectra were obtained in reflector mode with an acceleration voltage of 25 kV and a pulse ion extraction time of 80 ns. The mass range for MS was generally between 700 and 3500 m/z. The number of laser shots summed in MS was 3500. The number of laser shots summed in MS–MS was 3000. The software flexAnalysis (Bruker, Bremen, Germany) was used for peak picking prior to using the standard Mascot search engine. The peptide mass fingerprint was searched against the non-redundant Swiss-Prot protein database (all organisms) placed in the public domain by UNIProt and modified by the addition of sequences corresponding to the recombinant fusion proteins being studied in this manuscript. flexAnalysis takes advantage of the isotopic envelope available from high resolution MS–MS spectra in peptide identification. Mascot is available from Matrix Science Ltd, London, UK.

Electrospray LC–MS of intact proteins: Electrospray mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer. Samples were desalted on-line by reverse-phase HPLC on a Phenomenex Jupiter C4 column (5 μm, 300 Å, 2.0 mm × 50 mm) running on an Agilent 1100 HPLC system at a flow rate of 0.2 mL/min using a short water, acetonitrile, 0.05% trifluoroacetic acid gradient. The eluant was monitored at 214 nm and then directed into the electrospray source, operating in positive ion mode, at 4.5 kV and mass spectra recorded from 50–3000 m/z. Data were analysed and deconvoluted to give uncharged protein masses with Bruker's Compass Data Analysis software.

2.7. Site-specific mutagenesis

hGH mutants were made using the Agilent site-directed mutagenesis protocol. The primers used for the C53S mutation were (5′ to 3′): CCCCCAGACCTCCCTCTCTTTCTCAGAGTCTATTCCGAC and GTCGGAATAG ACTCTGAGAAAGAGAGGGAGGTCTGGGGG. For the C189S mutant the following primers were used: GTGGAGGGCAGCTCTGGCTTCCATCATC ATCATCATCAC and GTGATGATGATGATGATGGAAGCCAGAGCTGCCCTC CAC. For the double C53S C189S mutant, the C189S mutant was used as a template with the C53S primers (described previously.

3. Results

3.1. IFN, hGH, an scFv and a VH domain construct are all efficiently exported by Tat in the absence of prior disulfide formation

Previous studies [5,16] showed that several disulfide-bonded proteins, including PhoA, a phytase AppA, an scFv construct and a Fab fragment, were exported in *E. coli* when a Tat signal peptide was present at the N-terminus, provided that disulfide formation could occur in the cytoplasm. In one case this was achieved by expression in *Δgor/ΔtrxB* cells; this strain passively enables the formation of disulfide bonds in proteins in the cytoplasm by the removal of the two naturally occurring reducing pathways [23]. In the other case the proteins were expressed in 'CyDisCo' strains that express Erv1p and PDI in the cytoplasm, actively promoting efficient disulfide bond formation. The same constructs were not exported in wild type strains, with the Tat pathway clearly identifying these constructs as 'inappropriately folded' and rejecting them.

The primary aim of this study was to test whether the Tat system can export other disulfide-bonded therapeutic proteins with high efficiency, and we chose human growth hormone (hGH), human interferon α2b (IFN), an scFv raised against the omega peptide of β-galactosidase (described in [13]) and an antibody VH domain construct [7] as targets. We used constructs containing C-terminal His tags to aid identification of the proteins, and all proteins bore N-terminal TorA signal peptides (as used in [16]) to direct export by the Tat pathway. The precursor proteins were expressed on a pET23-based plasmid either alone or together with Erv1p and PDI.

Fig. 1 shows export assays using TorA-hGH and TorA-IFN. After a 3 h induction of expression, the cells were fractionated to yield cytoplasm, membrane and periplasm samples (C, M, P) which were immunoblotted using antibodies to the His tags on the target proteins. The figure shows that most of the TorA-IFN is exported to the periplasm (P) and processed to the mature size (20.7 kDa). A small amount of precursor protein is apparent in the membrane fraction and some mature-size protein is also present in the cytoplasm. This is commonly found during export of Tat substrates, probably because the signal peptide is prone to proteolytic clipping (see e.g. [15]). The export of TorA-hGH is even more efficient; the vast majority of protein is found in the periplasm and the precursor form is barely detectable in the cytoplasm.

Fig. 1 also shows similar assays carried out in wild type cells, i.e. in the absence of Erv1p/PDI. Surprisingly, export of both proteins is again highly efficient and both proteins are found predominantly in the periplasm as mature size proteins. Next, we tested for the export of the scFv and VH domain constructs, and export assays in wild type and CyDisCo

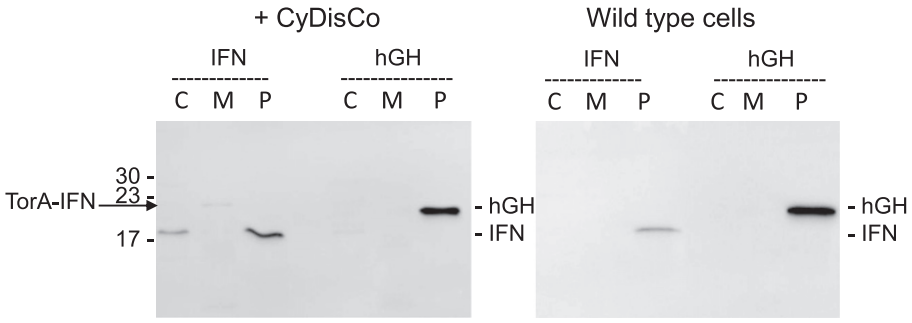

Fig. 1. Efficient Tat-dependent export of IFN and hGH by Tat in both the presence and absence of CyDisCo components. hGH and IFN were expressed with a TorA Tat signal peptide in *E. coli* in the presence or absence of the CyDisCo components Erv1p and PDI. After 3 h, cells were separated into cytoplasm/membrane/periplasm fractions (C, M, P) and samples were immunoblotted to detect the proteins. Mobilities of mature-size hGH and IFN are indicated, together with a faint TorA-IFN band in the '+CyDisCo' panel. The mobilities of molecular mass markers are indicated (in kDa) on the left.

strains are shown in Fig. 2. The data again show efficient export of both substrates, in that a substantial proportion of the scFv, and the majority of the VH domain construct, are found in the periplasm in both Erv1p/PDI-expressing and wild type cells. The TorA-scFv and TorA-VH precursor proteins are detected in the cytoplasm and membrane fractions, but a large proportion of scFv and most of the VH domain protein are present in the periplasm.

3.2. TorA-scFv is exported exclusively by the Tat pathway

The data shown in Figs. 1 and 2 are surprising because the Tat-dependent export of other disulfide-bonded proteins has shown an absolute dependence on prior cytoplasmic disulfide bond formation, as explained above. We therefore considered it possible that the substrates tested in this study may in fact be exported by the Sec pathway, since Sec-type signal peptides are similar in overall structural terms to Tat signal peptides. To test this possibility directly, we expressed some of the same constructs in *tat* null mutant cells and the data for TorA-scFv are shown in Fig. 3. The results show that the scFv is not exported in the *tat* mutant strain to any significant extent, either in the absence or presence of Erv1p/PDI; all of the protein is found in the spheroplast fraction (Sp). In control assays, export to the periplasm (P) is again observed in both wild type and CyDisCo strains, confirming that the export observed in Figs. 1 and 2 can be attributed solely to the Tat pathway. Identical results were obtained for the TorA-IFN and the TorA-VH domain protein (data not shown). These results are perhaps not surprising; in our previous study [16] we tested whether a different TorA-signal containing scFv was exported exclusively via Tat, by substituting the critical twin-arginine motif in the signal peptide with twin-lysine. The mutation totally blocked transport, confirming that the protein was transported by the Tat pathway.

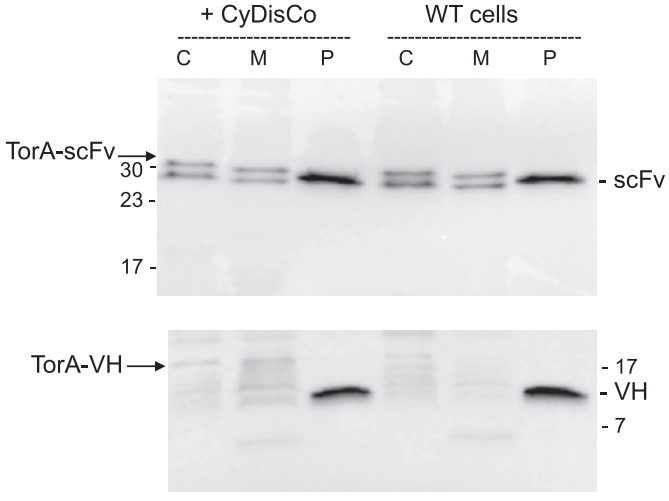

Fig. 2. scFv and VH domain antibody constructs are exported by Tat in the presence and absence of CyDisCo components. TorA-scFv and TorA-VH domain constructs were expressed in wild type or CyDisCo cells as described for IFN and hGH, and cells were fractionated to yield cytoplasm, membrane and periplasm samples (C, M, P). The samples were analysed by immunoblotting using antibodies to the His tag, and mobilities of TorA-scFv, mature scFv ('scFv'), TorA-VH domain protein and mature VH domain protein are indicated. Mobilities of molecular mass markers (in kDa) are indicated on the left (scFv panel) or right (VH domain panel).

3.3. TorA-scFv is efficiently exported and accurately processed to the mature size

The immunoblots shown in Figs. 1 and 2 show that the four proteins appear to be exported with high efficiency by the Tat pathway, in the sense that the majority of detected protein is in the periplasm. In order to assess the actual export flux, and to additionally test whether the proteins are correctly processed to the mature size, we analysed the samples by Coomassie staining of the periplasm sample and mass spectrometry of the exported scFv. Fig. 4A shows a Coomassie-stained gel of the periplasmic fractions after expression of TorA-scFv in wild type MC4100 cells or the *tat* null mutant strain, in either the presence or absence of Erv1p/PDI. The immunoblot (lower panel) shows that the scFv protein is present in the periplasm of the wild type cells and the Coomassie-stained gel (upper panel) shows that this corresponds to an abundant 28 kDa protein that is absent from the periplasm of the *tat* mutant strain. The presumed scFv band was excised and subjected to trypsin digestion and MALDI TOF as described in Materials and methods. This generated the peptide sequences shown in Fig. 4B which confirm the protein to be the scFv. Importantly, the N-terminal peptide sequences correspond precisely to the sequences immediately following the predicted signal peptidase cleavage site, with no evidence of incorrect processing or large-scale proteolytic clipping. These results

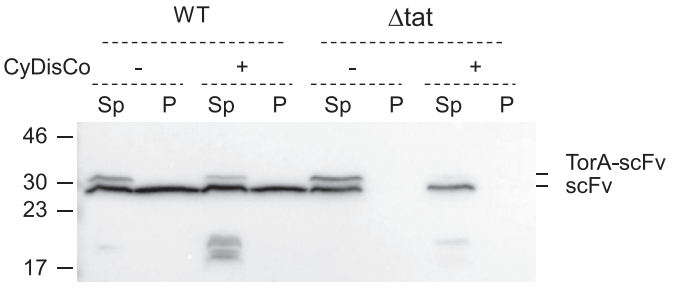

Fig. 3. Export of TorA-scFv occurs exclusively by the Tat pathway. TorA-scFv was expressed in wild type and *tat* null mutant strains for 3 h, in either the presence or absence of CyDisCo components as indicated. After this period cells were fractionated and spheroplast and periplasm (Sp, P) samples were analysed by immunoblotting using antibodies to the C-terminal His tag on the scFv protein. The mobilities of the precursor and mature scFv forms are indicated.

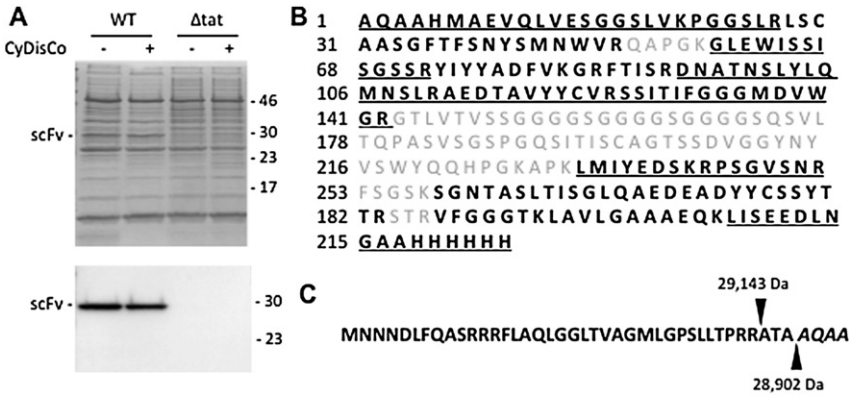

Fig. 4. TorA-scFv is efficiently exported and accurately processed. A: TorA-scFV was expressed in MC4100 cells or the *tat* null mutant strains in the presence or absence of CyDisCo components as indicated. The periplasmic fractions were run on Coomassie-stained gels and the putative scFv band (present in the periplasm of wild type cells but not *tat* null mutant cells) is indicated. Mobilities of molecular mass markers (in kDa) are indicated on the right. Lower panel shows immunoblot of same gel. B: the putative scFv band from the CyDisCo-expressing sample was excised and subjected to MALDI TOF for peptide mass fingerprinting. The full mature scFv sequence is shown with the peptides identified by MS in bold and the sequence identified by MS/MS in bold and underlined. C: the scFv protein from the cytoplasm was purified and analysed by Electrospray ionisation mass spectrometry. Two major peaks were obtained: the larger one (ca. 80% of total signal) showed an intact mass of 28,902 Da indicating that cleavage had occurred at the signal peptidase cleavage site (the mature protein sequence is italicised; see diagram). A second mass of 29,143 Da was also observed for a polypeptide in much lower abundance; this reflects cleavage after the arginine residue as shown.

provide strong evidence that the precursor protein is accurately processed to the mature size following export to the periplasm, and the relative abundance of the exported scFv, after only a 3 h export assay, shows that the protein is exported at a high rate in both the presence and absence of Erv1p/PDI.

It is notable in Fig. 4A that a high proportion of the cytoplasmic TorA-scFv is degraded to a form that co-migrates with authentic periplasmic mature scFv, and the data in Fig. 1 likewise show the presence of mature-size IFN in the cytoplasm. We analysed the cytoplasmic scFv form by mass spectrometry to identify the cleavage site(s) and the results are shown in Fig. 4C. In fact the masses of the proteins in the excised band show that the majority of the protein (about 80%) is indeed mature-size scFv, with a minor (20%) component cleaved after the last arginine in the signal peptide as shown. In principle, these results raise the possibility that cleavage is indeed carried out by signal peptidase; however, this is unlikely because its active site is on the periplasmic face of the plasma membrane and precursor proteins should not be able to reach this location in *tat* null mutant cells. Nevertheless, the frequent appearance of cytoplasmic mature-size protein in assays for Tat-dependent export raises interesting questions, and we cannot rule out the possibility that Tat signal peptides are indeed cleaved by this enzyme even in the absence of translocation. It may also be the case that other proteases cleave cytoplasmic precursor protein to peptides that are not detected: it is notable in Figs. 1 and 2 that the levels of TorA-hGH and TorA-IFN in the cytoplasm are extremely low, and this may not be due only to their export to the periplasm.

3.4. Tat-exported hGH and scFv acquire disulfide bonds in the periplasm

The 4 proteins tested in this study are clearly exported by Tat in the absence of disulfide bond formation, and we next tested whether one of the proteins acquires disulfide bonds in the periplasm. The DsbABCD system catalyses disulfide bond formation in the periplasm (reviewed in [12]), usually with proteins that have been exported by the Sec pathway in an unfolded state. In contrast, the Tat pathway almost certainly exports proteins in either a fully folded or near-native state, and under such circumstances it is by no means clear whether the Dsb system can access the required thiol groups in order to catalyse disulfide bond oxidation. This issue was investigated by analysis of the periplasmic proteins on reducing vs non-reducing SDS polyacrylamide gels; disulfide-bonded proteins usually migrate differently under non-reducing conditions because the presence of disulfide bonds prevents full SDS-dependent unfolding of the proteins.

Fig. 5A shows immunoblots of periplasmic hGH after export in Erv1p/PDI − expressing or wild type cells. The samples were run under reducing or non-reducing conditions (the latter samples were run in duplicate, in case reducing agent diffused to the adjacent lane). hGH run under non-reducing conditions clearly migrates faster than the fully reduced sample, confirming that this protein sample contains disulfide bonds. Importantly, the vast majority of the protein from both CyDisCo and wild type cells runs with this increased mobility (denoted 'hGH (ox)'), confirming the presence of disulfide bonds and providing strong evidence that hGH exported in wild type cells acquires

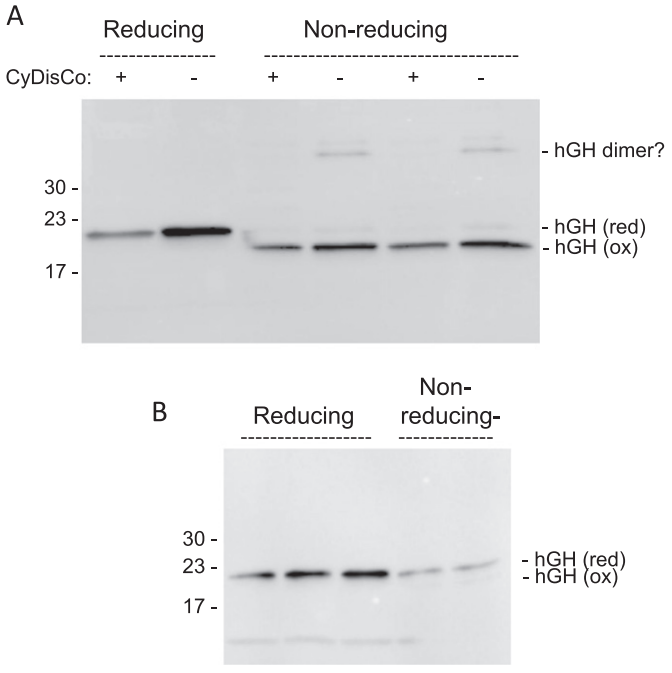

Fig. 5. Periplasmic hGH is disulfide-bonded after Tat-dependent export in wild type or CyDisCo-expressing cells. A: TorA-hGH was expressed in the presence or absence of CyDisCo components as indicated (+/−) and the periplasmic fraction was subjected to SDS-polyacrylamide gel electrophoresis in the presence or absence of reducing agent (2-mercaptoethanol) as indicated. The mobilities of reduced and oxidised hGH (red, ox) are indicated, as is a higher band that is possibly a dimer. B: the same TorA-hGH construct was expressed in a *dsbB*$^{-}$ strain in the absence of CyDisCo components, and samples of the periplasm fraction were again run under reducing or non-reducing conditions as in A. Mobilities of oxidised and reduced hGH forms are indicated (ox, red).

disulfide bonds in the periplasm. A small proportion of the protein runs further up the gel, possibly indicating formation of some dimer under these conditions.

It seemed likely that the exported, reduced hGH would acquire disulfide bonds using the DsbABCD machinery that catalyses disulfide bond formation in exported Sec substrates, and this was tested using the same approaches. TorA-hGH was expressed in a *dsbB*$^{-}$ strain that was previously used to study disulfide bond formation in other exported substrates [16] and the data are shown in Fig. 5B. In the absence of Erv1p/PDI, hGH is exported and the samples migrate as the usual 19 kDa band under reducing conditions ('hGH (red)'). Importantly, the samples still run primarily with the same mobility under non-reducing conditions, with only a minority migrating more rapidly like the oxidised form (hGH (ox)). We interpret these data to indicate that, in the absence of DsbB, the bulk of the exported hGH is unable to properly form disulfide bonds, strongly suggesting that the DsbABCD machinery catalyses the formation of the bonds in the experiment shown in Fig 5B. A minor proportion of exported hGH does appear to contain disulfide bonds, and we speculate that these may form as a result of spontaneous oxidation.

We also purified larger amounts of hGH from periplasmic samples after export in CyDisCo and wild type cells, and analysed the protein under reducing and non-reducing conditions. Fig. 6 shows that the purified hGH samples from CyDisCo and wild type cells both run as the expected 19 kDa band on reducing gels, whereas the faster-migrating form (containing disulfide bonds) is observed under non-reducing conditions. Importantly, the latter form predominates to an equal extent after purification from either wild type or CyDisCo cells, showing that the periplasmic protein can become essentially fully folded and disulfide-bonded whether the disulfide bonds are formed in the cytoplasm or periplasm. Taken together, the data from Figs. 5 and 6 indicate that the Tat system can export these proteins even when they lack disulfide bonds, and that these bonds can form after entry into the periplasm.

3.5. The Tat system can transport hGH even when disulfide bond formation is blocked by substitution of the Cys residues involved

The above studies imply that the Tat system is transporting a range of substrates in a reduced form, possibly because they are able to attain a near-native structure in the absence of disulfide bond formation. This is an important issue and we considered it important to confirm that a substrate can definitely be transported in the absence of prior disulfide bond formation. To do this we used site-specific mutagenesis to block disulfide bond formation in the hGH substrate. hGH contains two disulfide bonds; one between Cys53 and Cys165 (a long-range bond between residues remote from each other in sequence), and the other between Cys182 and Cys189 (a short range bond) (Fig. 7). Substitution of Cys53 by serine (variant C53S) prevents formation of the first bond whereas substitution of Cys189 by serine (C189S) blocks formation of the second. We tested for export of the C53S and C189S single mutants and the C53S/C189S double mutant, and the data are shown in Fig. 7; the positions of the above disulfide bonds in hGH are also illustrated in Fig. 7.

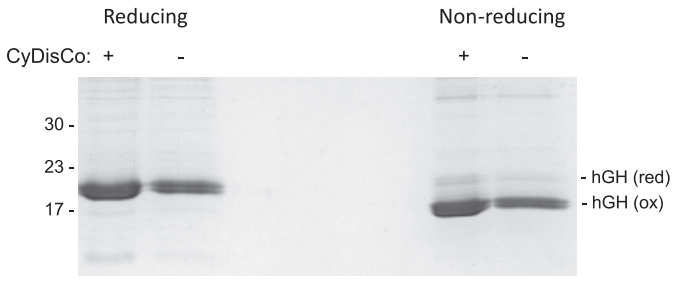

Fig. 6. hGH is almost completely disulfide-bonded after purification from the periplasm of wild type or CyDisCo-expressing cells. TorA-hGH was expressed in wild type or CyDisCo-expressing cells and the protein was purified from periplasmic extracts by IMAC chromatography as detailed in Materials and methods. The protein was run on SDS polyacrylamide gels in the presence or absence of 2-mercaptoethanol as indicated. Mobilities of oxidised and reduced forms are indicated.

The blot shows periplasmic samples from the export assays and the reduced samples (on the left) show the presence of very similar levels of exported periplasmic hGH in each case. None of the substitutions adversely affect translocation-competence. Analyses of the samples under non-reducing conditions are shown on the right hand side of the figure. The wild type protein shows the characteristic increase in mobility that accompanies disulfide bond formation, and the same increase in mobility is observed with the C189S mutant. On the other hand, the C53S and double mutant do not show this increase in mobility and instead migrate with the same mobility as the reduced samples. We conclude that the first disulfide bond (disrupted in C53S) is solely responsible for the shift in mobility in SDS polyacrylamide gels whereas disruption of the second bond (in C189S) does not have a significant effect. This is consistent with the protein structure (Fig. 7). The C182–C189 disulfide bond is a short-range bond whereas the C53S–Cys165 bond is a long range bond that effectively ties together distant regions of the protein. It is therefore unsurprising that the presence of this bond has a major effect on protein mobility when the sample is run under denaturing, non-reducing conditions. Notably, the Tat system exports all these variants and thus appears to recognise the proteins as correctly folded, despite the absence of one or both disulfide bonds.

4. Discussion

A number of previous reports [5,14,16] have shown that the Tat system has an effective, although poorly understood, quality control system that enables it to identify and reject proteins that are misfolded. This feature is important for the normal functioning of the system: many of its substrates are cofactor-containing proteins and it is important that these are only exported in a folded, assembled state. The feature does not only operate during the export of natural Tat substrates, however, since this selectivity operates towards a variety of heterologous proteins when expressed with Tat signal peptides, including some mammalian disulfide-bonded proteins.

The Tat pathway has considerable potential for the industrial production of biopharmaceuticals, since it is known to be capable of exporting a number of 'Sec-incompatible' proteins and is furthermore capable of exporting them at high rates in fermenter systems [15]. Moreover, the inbuilt quality control feature should enable the system to preferentially export proteins that are correctly folded, which is a potentially valuable trait for biotechnological applications. It was believed that the export of disulfide-bonded proteins would be problematic, since these cannot form native structures in the cytoplasm, but recent studies have shown that CyDisCo strains offer potential as a means of presenting the Tat system with prefolded, disulfide-bonded substrates that it can export to the periplasm [16]. The main aim of the present study was to determine whether this CyDisCo-Tat combination could be used for the production of other disulfide-bonded proteins, especially biotherapeutics.

We approached this issue by testing for export of human interferon α2b, hGH, an scFv and a VH domain construct. We show that all are exported by the Tat system with high efficiency; indeed, the export efficiencies observed with some of these substrates are higher than those we observe with many native Tat substrates, despite expressing these heterologous substrates at relatively high levels using pET23-based plasmids. However, the major surprise from this study is that none of these substrates require prior disulfide bond formation for export; the proteins are all exported with similar, usually undiminished efficiencies in wild type cells where the reducing cytoplasm prevents disulfide bond formation prior to export. The likely explanation is that the

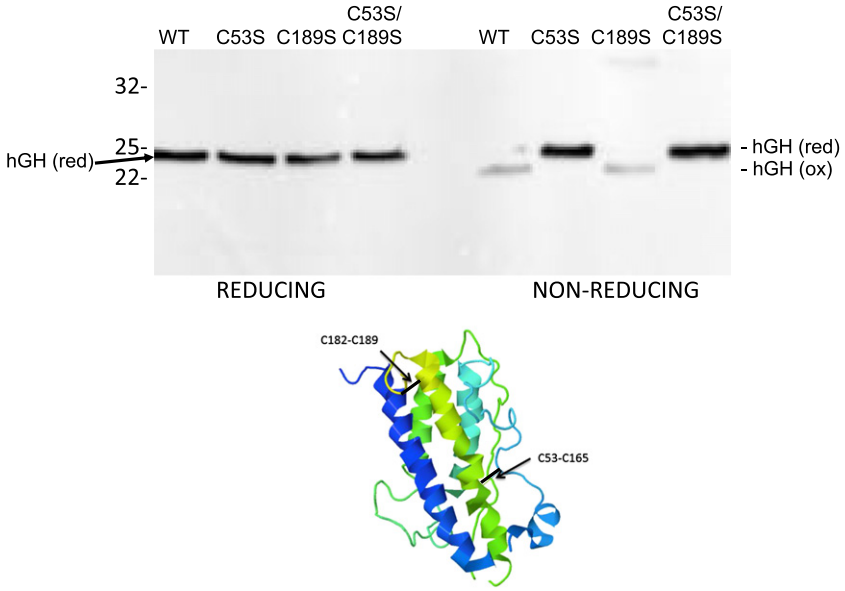

Fig. 7. Complete disruption of one or both disulfide bonds in hGH does not affect export by the Tat pathway. Ribbon diagram of the hGH structure showing the positions of the disulfide bonds (between C53 and C165, and C182–C189). Site-specific mutagenesis of TorA-hGH was carried out to substitute one of the Cys residues involved in the first or second disulfide bond (Cys53, Cys189, respectively) by serine. A third, double-mutant combined both substitutions. All 3 variants were expressed in *E. coli* W3110 cells, together with non-mutated TorA-hGH (WT), and the periplasmic fractions were isolated. These samples were run on SDS-polyacrylamide gels under reducing (left) or non-reducing (right) conditions. Mobilities of oxidised and reduced hGH are indicated.

proteins adopt structures that are sufficiently native-like to be accepted by the Tat system's proofreading system. Indeed, one of the substrates, hGH, is known to adopt a near-native structure in the absence of disulfide bond formation, and is even active in this state [3,30].

These experiments were carried out in a very similar manner to those described in Matos et al. [16] and the question is why this set of substrates shows such differing requirements for export. We believe that the most likely reason relates to the folding complexity of the proteins studied in those previous reports by DeLisa et al. [5] and Matos et al. [16]. PhoA is a 51 kDa protein with two sequential disulfide bonds, while AppA is a 53 kDa protein with 4 disulfide bonds. The dimeric Fab construct studied in the DeLisa et al. [5] report is particularly complex with both intra-chain and inter-chain disulfide bonds. In contrast, the proteins tested in the present study are smaller, with molecular masses of 20.7 kDa (IFN), 23.6 kDa (hGH), 28.9 kDa (scFv) and none contain more than 2 disulfide bonds. One possibility is that the simpler 3-dimensional structure of the proteins enables them to acquire native, or near-native structures in the absence of disulfide bond formation. However, we would emphasise that this scenario may well be too simplistic, and in our view it is likely that other factors dictate whether the Tat-dependent export of such proteins is reliant on prior disulfide formation, and hence on the CyDisCo components Erv1p and PDI.

Finally, the data have implications for the mechanism of the Tat proofreading system. While this system has a remarkable ability to detect whether substrates are folded, it is clearly not perfect and the 4 substrates analysed in this study were able to 'fool' the translocation pathway and undergo transport. The first point is that the Tat system clearly cannot detect whether disulfide bonds have formed in substrates, at least in these proteins. The second point is that, assuming that all 4 substrates are not 100% correctly folded in the absence of disulfide bond formation, there appears to be some margin for error in the proofreading mechanism. It will be interesting to determine just how close to 'native' such Tat substrates' structures need to be, in order to undergo translocation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2014.12.027.

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