Sortase-modified cholera toxoids show specific Golgi localization

Darren C. Machin\[a\], Daniel J. Williamson\[a\], Peter Fisher\[b\], Victoria J. Miller\[c\], Gemma C. Wildsmith\[a\], James F. Ross\[a\], Christopher Wasson\[d\], Andrew Macdonald\[d\], Benjamin I. Andrews\[e\], Daniel Ungar\*\[b\], W. Bruce Turnbull\*\[a\] and Michael E. Webb*\[a\]

Abstract: Cholera toxoid is an established tool for use in cellular tracing in neuroscience and cell biology. We use a sortase-labelling approach to generate site-specifically N-terminally modified variants of both the A2-B5 heterohexamer and B5 pentamer forms of the toxoid. Both forms of the toxoid are endocytosed by GM1-positive mammalian cells, and while the heterohexameric toxoid was principally localized in the ER, the B5 pentamer showed an unexpected localization in the medial/trans Golgi. This study suggests a future role for specifically-labelled cholera toxoids in live-cell imaging beyond their current applications in neuronal tracing and labelling of lipid-rafts in fixed cells.

Introduction

Cholera toxin and the closely related heat-labile enterotoxin share an identical hexameric AB5 structure composed from five B-subunits (B5) arranged in a pentamer around a central A-subunit (Figure 1)[1]. The B5 pentamer can bind to up to five GM1-gangliosides on the surface of mammalian cells to create a powerful multivalent effect that triggers endocytosis of the toxin[2]. Once internalized, a short KDEL targeting sequence located at the C-terminus of the A-subunit is thought to direct the protein to the endoplasmic reticulum (ER) via the trans-Golgi network (TGN)[3]. The peptide chain between the A1 and A2 subunit is cleaved in the TGN and upon arrival in the ER the A1-domain is released from the rest of the toxin by protein disulfide isomerase (PDI) and transported through the Sec61 channel into the cytosol[4]. ADP-ribosylation factor-6 binds to the A1-protein in the cytosol allowing it to reach its target destination, a G-protein, and induce toxicity[5]. Importantly, the retrograde trafficking mechanism used by the bacterial toxins to gain entry into cells is active without the catalytic A1-domain or A-subunit.

The intrinsic ability of the bacterial toxins to induce endocytosis has been exploited in several studies to transport biomolecules and probes into mammalian cells both in vivo and in vitro[8]. Intracellular delivery has predominantly been achieved through the use of bacterial toxin chimeras, but this approach requires a gene construct to be created for every novel protein fusion and it is quite likely that expression optimisation would also be necessary. Relatively recently, sortase A (SrtA) has been used to attach a selection of chemical probes and a protein to the A1-domain of a cholera toxin analogue allowing the intracellular fate of the protein to be studied in more detail[7]. However, the analogue retained its toxic action, so its application as a general delivery system is limited. In a separate study, the N-terminus of the B-subunit was also labelled using SrtA-mediated ligation, but the ability of the modified proteins to form stable pentamers and enter mammalian cells was not established[8]. In this work we sought to both generate non-toxic A2-B5 and B5 toxoids and to confirm the localization of these proteins in mammalian cells.

SrtA is a type II membrane-bound protein that “sorts” and covalently anchors virulence factors to the cell wall of Gram-positive bacteria[9]. The enzyme binds to proteins carrying an LPXTG recognition motif and attaches them onto peptidoglycans bearing an N-terminal oligoglycine sequence. This ligation mechanism has been used in numerous studies[9,10,11] to label the

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\[a\] Dr Darren C. Machin, Dr Daniel J. Williamson, Dr James F. Ross, Professor W. Bruce Turnbull, Dr Michael E. Webb School of Chemistry and Astbury Centre for Structural Molecular Biology University of Leeds, Leeds, LS2 9JT E-mail: w.b.turnbull@leeds.ac.uk, m.e.webb@leeds.ac.uk

\[b\] Dr Peter Fisher, Dr Daniel Ungar Department of Biology, University of York, York, YO10 5DD E-mail: dani.ungar@york.ac.uk

\[c\] Dr Victoria J. Miller Department of Biochemistry, University of Bristol, Bristol

\[d\] Dr Christopher Wasson, Dr Andrew Macdonald Faculty of Biological Sciences and Astbury Centre for Structural Molecular Biology University of Leeds, Leeds LS2 9JT

\[e\] Dr Benjamin I. Andrews, GlaxoSmithKline, Medicines Research Centre, Gunnels Wood Road, Stevenage, Hertfordshire SG1 2NY, United Kingdom
N- and/or C-terminus of proteins with a range of chemical probes including biotin, fluorescein and cholesterol. The advantage of SrtA-mediated ligation over other protein-labelling techniques is that it only requires the introduction of a single sterically accessible glycine residue at the N-terminus of a protein substrate or an LPXTGX motif at the C-terminus. In addition, the use of depsipeptide substrates, previously developed by our group, can greatly increase the N-terminal labelling efficiency\[11\]

Results and Discussion

We took two approaches to generate modified cholera toxins, based either on single labelling of a truncated A2-B5 toxoid or multiple functionalization of the B5 subunit alone. For the A2-B5 cholera toxoid, we generated an expression construct in which the coding sequence for a maltose binding protein-A2 fusion protein is 5’ to that for the B subunit in a polycistronic construct under the control of the lac promoter in an adapted pMalc5x backbone (see supplementary information). The expressed proteins are both co-translationally exported into the periplasm before assembly and export into the growth medium. While traces of the fusion protein could be detected in both the periplasm and the growth medium, we isolated it from the growth medium via ammonium sulfate precipitation followed by sequential amylose and immobilized metal affinity chromatography to yield the protein complex. Treatment of the MBP-A2-B5 construct with TEV protease yielded an N-terminal triglycine motif on the A2 subunit. Using our reported sortase labelling approach, after optimisation this was quantitatively labelled using a three-fold excess of a FITC-depsipeptide and 10 mol% sortase (figure 2) followed by purification by size-exclusion chromatography. If the samples are not boiled, both the B5 pentamer and the A2-B5 complex are sufficiently stable to electrophorese as multimers in SDS-PAGE. Unlabelled protein is not observed in the SDS-PAGE analysis and although trace peaks corresponding to the unlabelled protein (figure 2C, blue spectrum) can be detected via ES-MS these are similar in intensity to other minor contaminating species.

The trafficking and localization pattern of the FITC-A2-B5 was initially studied in monkey epithelial kidney (B-SC-1) cells. The cells were incubated with 150 nM A2-B5 complex in medium and before acid-washing to remove surface-bound proteins, fixing with MeOH, counterstaining with antibodies to calnexin (ER) and GM130 (cis-Golgi) marker proteins and wide-field fluorescence imaging (figure 3A). After 5 minutes of uptake, the protein was largely observed in small punctate bodies consistent with endocytic vesicles (not shown). After 30 minutes, the protein was located diffusely throughout the cell with some concentration in a perinuclear body consistent with localization in both the ER (shown by colocalization with the calnexin marker) and the Golgi. After 60 minutes, the protein was principally concentrated in a perinuclear location consistent with the Golgi, adjacent to but not coincident with the GM130 cis-Golgi marker. This observation of trafficking to the ER and to Golgi-associated membrane bodies is consistent with previous observations of cholera toxin trafficking. The observation of the protein largely in a Golgi-associated compartment after an hour rather than the ER was unexpected. We therefore repeated the experiment (in HEK293 cells, figure 3B) using a pulse-chase approach in which the A2-B5 toxin was initially added to the cells before washing and further 2.5 h incubation in growth medium (figure 3B). In this case, a mixed

Golgi/ER localization is observed. This suggests that the capacity of the cell to traffic the A2-B5 from the cell surface to the Golgi is substantially greater than the capacity of its KDEL-linked transport to the ER.
Following labelling of the A2-B5 system, we sought to use the same approach to modify the simpler cholera toxin B homopentamer to determine whether we would observe the same transport to the Golgi. Naturally-occurring cholera toxoids have either an N-terminal alanine or threonine. While the latter isoform can be labelled using periodate-mediated oxidation and imine formation[13] we wished to use the more chemically stable peptide formed as a result of labelling using sortase. Ploegh and co-workers have previously demonstrated that a triglycine extension is required for efficient labelling of this scaffold [8]. We therefore generated Cholera toxin B with an N-terminal triglycine extension (GGG-CTB). Briefly, the coding sequence was generated using assembly PCR[13] and subcloned into a variant of pMALp5x in which the malE gene has been replaced with the LTB-leader sequence for periplasmic expression. Protein was overexpressed in E. coli C41 (DE3) cells and purified from the growth medium by sequential ammonium sulfate precipitation, immobilized metal-affinity chromatography and size-exclusion chromatography. The protein was subsequently labelled for four hours using 2.5 equivalents of a FITC-depsipeptide and 10 mol% sortase to generate FITC-CTB (figure 4A & B). Analysis of the labelling reaction by SDS-PAGE and ES-MS indicated >90% labelling of the protein.

While CTB has previously been labelled using sortase, the functional consequences of this labelling have not been assessed. Unlike the N-terminal of the A2 subunit, the N-terminus of CTB is adjacent to the GM1 binding site and it was therefore possible that addition of the eight amino-acid linker and FITC might have an adverse effect on either ligand binding or cell entry. We assayed GM1 ganglioside binding affinity and stoichiometry using ITC (table 1 and supplementary information). GGG-CTB bound GM1 in a 1:1 ratio consistent with wt-CTB while the labelled FITC-CTB exhibited stoichiometric binding but with a small reduction in affinity. We next investigated the effect of labelling on cell-trafficking by the toxin. We used Vero (monkey kidney epithelial) cells as a convenient system to study endocytosis. After 2 hours...
incubation, the labelled protein was localized to a single region of punctate spots consistent with localization to the Golgi (figure 4C). To confirm that this labelling pattern was due to intact FITC-CTB and that peptidic cleavage of the linker sequence had not occurred we carried out immunofluorescent co-staining with an anti-CTB (antibody and confirmed that the FITC-labelling was colocalized with CTB (supplementary figure 1).

Endocytosis of the intact cholera toxin has been shown to be mediated by a wide variety of potential pathways, and it is thought to be translocated to the ER via the trans-Golgi network. We therefore investigated both the time taken to establish the stable labelling pattern (see supplementary figure 2) and the precise location of the protein in both Vero and HEK293 (human embryonic kidney) cells. In Vero cells FITC-CTB could be detected as disperse puncta throughout the cell after 5 minutes of uptake in both wide-field and confocal imaging, after approximately 30 minutes localization to the same distinct perinuclear region as observed for the A2-B5 toxoid is apparent and this localization is essentially complete after approximately 1 hour (see supplementery information). Co-staining with an antibody to RCAS1, a trans-Golgi marker, showed some colocalization suggesting that the protein is largely resident in a Golgi compartment. Continued cell-growth had no effect on the distribution of the protein. In the human cells, a similar pattern was observed (see figure 5A). Importantly, no ER staining was detected despite using the same pulse chase approach as done with A2-B5 in the HEK293 cells. The morphology of the labelling pattern was again consistent with labelling of the Golgi and we therefore used immunostaining to identify the compartment. In human cells, the labelled portion of the cell is consistently between the GM130 cis-Golgi marker and the TGN46 TGN marker, suggesting the protein is localized to either the medial- or trans-Golgi. We therefore used nocodazole treatment to induce formation of Golgi mini-stacks, which allowed more precise localization of CTB relative to the cis-Golgi and TGN markers (figure 5D). Following addition of CTB construct for 30 min, the cells were incubated in growth medium for 2.5 or 24 hours followed by a 3 hour treatment with 5 µM nocodazole before cell fixation and imaging by immunofluorescence. The signal for FITC-CTB is always observed between that for the trans- (TGN46, violet) and cis-Golgi (GM130, red) markers consistent with a medial-Golgi localization.

Table 1. Thermodynamic parameters for CTB binding to GM1 ganglioside determined by isothermal titration calorimetry.

<table>
<thead>
<tr>
<th>Protein</th>
<th>N</th>
<th>Kd</th>
<th>ΔG</th>
<th>ΔH</th>
<th>ΔS</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CTB</td>
<td>0.93</td>
<td>60±20</td>
<td>-41.2±0.9</td>
<td>-57.7±1.7</td>
<td>-16.6±0.6</td>
</tr>
<tr>
<td>GGG-CTB</td>
<td>0.93</td>
<td>90±20</td>
<td>-40.2±0.5</td>
<td>-58.2±2.1</td>
<td>-18.0±0.7</td>
</tr>
<tr>
<td>FITC-CTB</td>
<td>1.08</td>
<td>358±62</td>
<td>-36.7±0.4</td>
<td>-69.0±4.6</td>
<td>-32.2±2.2</td>
</tr>
</tbody>
</table>

GM1 it is possible to induce CTB endocytosis by addition of GM1-ganglioside to cell culture. We used this approach for the COG4 knock out HEK293 cell line to investigate whether the COG complex was also required for CTB transport to the medial-Golgi. In all cases, addition of GM1 to the cell culture restored CTB
uptake, and the final distribution of protein within the cell was essentially identical to that observed in the parental HEK293 cell line (figure 5C). The time taken to establish this localization pattern was, however, delayed (see supplementary information), confirming that the COG complex is not essential for retrograde transport of the toxoid, but required for efficient transport.

Conclusions

In conclusion, we have generated two forms of labelled cholera toxoids using sortase-mediated labelling to generate quantitatively labelled proteins. While the A2-B5 toxoid shows a mixed pattern of localisation between the ER and Golgi presumably due to KDEL-related shuttling, the B2 toxoid accumulates in the medial–GolgI. This localisation is not reliant upon the major protein complex controlling retrograde trafficking of proteins in the Golgi though the efficiency is decreased in its absence. This suggests that the CTB toxoid has potential applications in both live cell-imaging and in controlled delivery to the medial Golgi.

Experimental Section

Protein overexpression and purification MBP-A2-Bs was overexpressed from vector pSAB2.1 in E. coli C41 (DE3) cells. The complex was isolated from the growth media by sequential amylase affinity, Ni-NTA affinity and size-exclusion chromatography. CTB and GGG-CTB were overexpressed from vectors pSAB2.2 and pSAB2.2(GGG-CTB) in E. coli C41 (DE3) cells. MBP-TEV was overexpressed from vector pMalc5x(TEV) in E. coli BL21-Gold (DE3). WT-SrtA was overexpressed from vector pET28a(SrtA) as described previously[11b].

Protein labelling Dnsyl and FITC-depsipeptides were synthesised using the Fmoc strategy for solid-phase peptide synthesis as previously reported[11b]. Individual protocols for protein labelling are described in the supplementary information.

Cell culture and fluorescence imaging Vero and HEK293 cells were cultured using standard protocols in DMEM supplemented with 10% FBS and 2 mM Gln, after incubation with the protein, cells washed with ice-cold 0.5 M Gly pH 2.2, fixed with ice-cold aqueous methanol and imaged using an Improvision 3DM wide-field imaging system. Following pulse chase with protein HEK293 cells were fixed with 1% paraformaldehyde, stained as described in the supplementary information and visualised using an LSM Protein HEK293 cells were fixed with 3% paraformaldehyde stained as described in the supplementary information and visualised using an LSM 880 Airyscan microscope (Zeiss).

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