Clathrin-dependent trafficking of subtilase cytotoxin, a novel AB₅ toxin that targets the endoplasmic reticulum chaperone BiP

Damien C. Chong,1 James C. Paton,1 Cheleste M. Thorpe2 and Adrienne W. Paton1*

1School of Molecular and Biomedical Science, University of Adelaide, SA 5005, Australia.
2Division of Geographic Medicine and Infectious Diseases, Tufts-New England Medical Center, Boston, MA 02111, USA.

Summary

Subtilase cytotoxin (SubAB) is the prototype of a new family of AB₅ cytotoxins produced by Shiga toxigenic Escherichia coli. Its cytotoxic activity is due to its capacity to enter cells and specifically cleave the endoplasmic reticulum (ER) chaperone BiP. However, its trafficking within target cells has not been investigated previously. In Vero cells, fluorescence colocalization with subcellular markers established that SubAB is trafficked from the cell surface to the ER via a retrograde pathway similar, but not identical, to those of Stx and Ctx, with their pathways converging at the Golgi. The clathrin inhibitor phenylarsine oxide prevented SubAB entry and BiP cleavage in SubAB-treated Vero, HeLa and N2A cells, while cholesterol depletion did not, demonstrating that, unlike either Stx or Ctx, SubAB internalization is exclusively clathrin-dependent.

Introduction

Pathogenic bacteria deploy a diverse array of toxins in order to damage their hosts, and as a consequence, cause massive global morbidity and mortality in animals and humans. The different types of toxins include membrane-damaging toxins, super-antigens and AB toxins. The latter group are characterized by B subunits, which recognize and bind to specific receptors on the surface of eukaryotic cells and direct the internalization of an enzymatic A subunit. Typically, the substrates for the various A subunits are essential components of the host cell biosynthetic or regulatory machinery located in the cytosol, necessitating translocation of the A subunit across a membranous barrier in order to engage its target. For toxins such as anthrax and diphtheria toxin, cell entry involves internalization into endosomes, with the low pH of this compartment triggering direct translocation of the enzymatic component of the toxin into the cytosol (Sandvig and van Deurs, 2002). For the so-called AB₅ toxins such as Shiga toxin (Stx) and cholera toxin (Ctx), trafficking is more complex. After binding to their respective glycolipid receptors via pentameric B subunits, they are internalized by receptor-mediated endocytosis. The toxin–receptor complexes then enter a retrograde transport pathway and traffic via the trans-Golgi network (TGN) and Golgi to the endoplasmic reticulum (ER) (Sandvig et al., 1992; Sandvig and van Deurs, 2002; Lencer and Tsai, 2003). The B subunits of both Stx and Ctx are essential and sufficient for this trafficking. Retrotranslocation of the A subunits from the ER lumen to the cytosol is then achieved by subversion of the protein-translocation channel Sec61 (Lencer and Tsai, 2003; Yu and Haslam, 2005).

Subtilase cytotoxin (SubAB) is the prototype of a new AB₅ toxin family that was discovered in a strain of Shiga toxigenic Escherichia coli (STEC) responsible for an outbreak of haemolytic uremic syndrome (HUS). HUS is a life-threatening complication of STEC disease, which is characterized by a triad of microangiopathic haemolytic anaemia, thrombocytopenia and renal failure. These clinical manifestations have long been considered to be directly attributable to effects of Stx on endothelial cells (Paton and Paton, 1998). The new toxin was named ‘subtilase cytotoxin’ because its 35-kDa A subunit (SubA) shares sequence homology to a subtilase-like serine protease of Bacillus anthracis (Paton et al., 2004). Subtilases are found in a wide variety of microorganisms, but none had previously been shown to be cytotoxic, nor have any been shown to be associated with a B subunit (Siezen and Leunissen, 1997). Mutagenesis of a critical Ser residue in SubA abolished toxicity, indicating that serine protease activity is central to the mechanism of action of the holotoxin (Paton et al., 2004). The B subunit (SubB) is related to putative exported proteins from Yersinia pestis and Salmonella Typhi, and mediates binding to the surface of target cells. SubAB is more toxic for Vero

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(African green monkey kidney) cells than Stx in vitro, and is lethal for mice (Paton et al., 2004). Remarkably, we have recently shown that intraperitoneal injection of mice with purified toxin results in pathological features that overlap those of Stx-induced HUS (Wang et al., 2007). The production of two potentially lethal cytotoxins by a single pathogen raises interesting questions about the relative importance of each toxin to human disease pathogenesis, and whether they might act in synergism.

Recently we have reported that the extreme cytotoxicity of SubAB for eukaryotic cells is due to a specific single-site cleavage of BiP (also known as GRP78) (Paton et al., 2006). BiP is an Hsp70 family chaperone located in the ER, and comprises an N-terminal ATPase and a C-terminal protein-binding domain. It mediates correct folding of nascent secretory proteins, but BiP is also responsible for maintaining the permeability barrier of the ER membrane by sealing the lumenal end of the Sec61 translocon pore, as well as for targeting of terminally misfolded proteins to the Sec61 apparatus for degradation by the proteasome (Gething, 1999; Hendershot, 2004). BiP plays a crucial role in the unfolded protein response as the ER stress-signalling master regulator, as well as exhibiting anti-apoptotic properties through interference with caspase activation. (Kim and Arvan, 1998; Rao et al., 2004). SubAB cleaves BiP at a dileucine motif in the hinge connecting the ATPase and protein-binding domains (Paton et al., 2006), and such disruption of BiP function has inevitably fatal consequences for the cell (Hamman et al., 1998; Kim and Arvan, 1998; Rao et al., 2004; Lee, 2005). SubAB exhibits extraordinary specificity for a subtilase-like serine protease, and it does not appear to cleave any other host cell protein, including the most closely related chaperones Hsp70 and Hsc70. This specificity may be explained by the fact that the catalytic site of SubA is partially occluded and lies at the bottom of a deep cleft (Paton et al., 2006). SubAB is the only toxin known to target a eukaryotic chaperone protein or a component of the ER. It is also the only AB toxin whose specific substrate is not located in the cytosolic compartment. For this reason, we have investigated the uptake and trafficking of SubAB within target cells, and compared its behaviour with that of Ctx and Stx.

Results

Co-trafficking of SubAB with Ctx and Stx

To investigate the uptake and intracellular trafficking pathway of SubAB, the transport of fluorescent SubAB in Vero cells was initially compared with that of other AB5 toxins by confocal fluorescence colocalization. The retro-translocation pathways of Stx (Sandvig et al., 1992) and Ctx holotoxins (Lencer and Tsai, 2003) have been previously characterized and shown to be identical to that of their respective B pentamers (Fujinaga et al., 2003; Khine et al., 2004). When Vero cells were coincubated with Oregon Green-labelled SubAB (SubAB-OG) and the B subunits of either Stx or Ctx labelled with Texas Red or Alexa Fluor 594, respectively (StxB-TR or CtxB-AF594), there were marked differences in tropism for individual cells (Fig. 1). Some cells bound and internalized SubAB or CtxB or StxB alone, while others took up both or neither of the toxins to which they were exposed. For Vero monolayers treated with Oregon Green-labelled SubAB (SubAB-OG) and the B subunits of either Stx or Ctx labelled with Texas Red or Alexa Fluor 594, respectively (StxB-TR or CtxB-AF594), there were marked differences in tropism for individual cells (Fig. 1). Some cells bound and internalized SubAB or CtxB or StxB alone, while others took up both or neither of the toxins to which they were exposed. For Vero monolayers treated with SubAB-OG and StxB-TR, examination of multiple fields (> 100 cells examined) indicated that 20.7% of the cells bound SubAB alone, 16.2% bound StxB alone, 35.2% bound both toxins, and 27.9% remained unstained. For monolayers treated with SubAB-OG and CtxB-AF594, 18.9% of cells bound SubAB alone, 47.8% bound CtxB alone, 27.0% bound both toxins, and 6.3% remained unstained. In cells that simultaneously internalized two toxins, fluorescence colo-
calization was prevalent in juxtanuclear regions and occasional puncta. However, there was also evidence of differential staining of puncta demonstrating that while intracellular trafficking pathways appeared to be similar for SubAB and CtxB (63.2% overlap) or StxB (64.2% overlap), they were not identical.

To address the possibility of the fluorochrome altering cell specificity or trafficking of SubAB, Vero cells were coincubated with SubAB-OG and SubAB-TR. The two species were observed to completely colocalize, demonstrating that their cellular specificity and trafficking pathways were identical (data not shown). Furthermore, the enzymatic activity of SubA did not affect cellular uptake or trafficking, as incubation of Vero cells with the inactive mutant toxin SubA_{A272B} labelled with OG resulted in the same staining pattern as SubAB-OG (data not shown). Likewise, subcellular transport of SubB-OG was identical to that of SubAB-OG (data not shown), confirming that the presence or absence of the A subunit has no influence on trafficking.

Fluorescence colocalization with subcellular markers
To further examine the trafficking route of SubAB within Vero cells, colocalization of SubAB-OG with various fluorescent organelle markers was examined after 30 min exposure to toxin and a 30 min chase (Fig. 2). SubAB-containing vesicles colocalized with the endosome marker TR-conjugated lysine fixable 70 000 MW dextran (Dextran-TR; 88.7% overlap), and TR-labelled transferrin (TF-TR) (56.1% overlap). However, only occasional colocalization of SubAB-TR with caveolae was observed in transfected Vero cells expressing Caveolin-1-EGFP (3.7% overlap). Similarly, transport of SubAB-OG to lysosomes labelled with LysoTracker DND-99 was rarely observed (2.0% overlap). This was not unexpected, since trafficking of AB toxin to lysosomes results in toxin degradation, and typically occurs only in non-sensitive cell types.

Translocation via the TGN and Golgi was confirmed by colocalization with TR-labelled wheat germ agglutinin (WGA-TR; 93% overlap). Similarly, immunofluorescent labelling of Golgin-97 (a membrane protein localized to the Golgi’s cytoplasmic face (Verma et al., 2000)) in Vero cells exposed to SubAB-OG revealed translocation to the juxtanuclear Golgi apparatus (88.7% overlap). Co-localization with immuno-labelled BIP showed toxin association with its intracellular substrate and by inference, its transport to the ER compartment (32.5% overlap). Trafficking to the ER was also confirmed by directly labelling the ER with ER-Tracker Red in cells exposed to SubAB-OG (27.7% overlap). Co-localization of the toxin with mitochondria labelled with MitoTracker CMXRos was not observed (~1% overlap).

The colocalization of SubAB-OG with TF-TR observed above raised the possibility that the toxin might be internalized via a clathrin-dependent endocytic pathway. To investigate this, early stages in trafficking were examined using a subset of the subcellular markers used above (Fig. 3). After 5 min incubation, there was significant colocalization of SubAB-OG with TF-TR (40% overlap); TF-TR is a marker for clathrin-coated vesicles at such early time points. However, SubAB-OG did not appear to colocalize with anti-Golgin-97 or anti-BIP (both exhibited ~1% overlap) at 5 min, indicating that detectable levels of toxin had yet to arrive at either the Golgi or the ER respectively.

Inhibition of trafficking
Further information on the intracellular trafficking pathway of SubAB in comparison with CtxB and StxB was obtained by subjecting Vero cells to a variety of pretreatments reported to obstruct retrograde transport. Cells were then exposed to SubAB-OG, CtxB-AF594 or StxB-TR for 3 h and examined for evidence of disruption of trafficking (Fig. 4). To establish if SubAB-OG was internalized by a passive or an active mechanism, cells were incubated at 4°C to diminish host cell metabolic activity. Binding of SubAB-OG to the Vero cell surface was independent of the incubation temperature. However, unlike control cells grown at 37°C, those incubated at 4°C did not internalize SubAB-OG. Lowering the cytosolic pH with media supplemented with acetate is known to prevent invagination of the plasma membrane and subsequent vesicle formation (Khine et al., 2004). SubAB-OG accumulated on the surface of cells treated with 60 mM acetate. These data indicate that in normal cells, SubAB is actively internalized within membrane-bound vesicles. Similar effects were observed for CtxB-AF594 and StxB-TR (Fig. 4).

Targeting of vesicles containing SubAB-OG to distinct Golgi was no longer apparent in the presence of the microtubule depolymerizer nocodazole (De Brabander et al., 1976), which suggests SubAB-OG may exploit a microtubule-dependent endosomal pathway. Similar redistributions were also noted with CtxB-AF594 and StxB-TR in nocodazole-treated cells (Fig. 4). However, nocodazole has been reported to disrupt and disperse the Golgi apparatus (Lippincott-Schwartz et al., 1990), and trafficking of SubAB-OG to the fragmented Golgi continued in the presence of nocodazole, as demonstrated by fluorescence colocalization with immuno-labelled Golgin-97 (result not shown).

Treatment with filipin, which binds cholesterol and inhibits caveola formation (Rothberg et al., 1992), did not prevent SubAB-OG uptake, indicating that transport from the plasma membrane was not entirely caveola-dependent. However, filipin clearly affected cell viability and morphology potentially disrupting Golgi targeting.
To investigate this further, cells were depleted of cholesterol by pretreatment with genistein and/or methyl-β-cyclodextrin (MβCD) (Le and Nabi, 2003); these treatments had no effect on cell viability at the doses and incubation times employed. Genistein and MβCD, singly or in combination, had minimal impact upon SubAB-OG trafficking. In contrast, MβCD caused marked surface accumulation of CtxB-AF594, while both MβCD and genistein affected targeting of StxB-TR to the Golgi (Fig. 4).
Oregon Green-labelled SubAB accumulated on the surface of Vero cells pretreated with the clathrin inhibitor phenylarsine oxide (PAO), indicating that endocytosis of SubAB-OG is mediated by a strictly clathrin-dependent pathway. In contrast, PAO did not block internalization of either CtxB-AF594 or StxB-TR, although there was some perturbation of retrograde transport in both cases (Fig. 4). In a separate experiment, Tf-TR also accumulated on the surface of PAO-treated Vero cells, and there was extensive colocalization with SubAB-OG (86.7% overlap) (Fig. 5). This confirmed that under our experimental conditions, PAO is a specific inhibitor of clathrin-dependent internalization.

The Golgi-disrupting agent brefeldin A (BFA) reversibly fuses endosomes with the TGN and the trans-, medial- and cis-Golgi cisternae with the ER (Lippincott-Schwartz et al., 1991; Donta et al., 1993). In this study, BFA treatment caused all three labelled toxins to accumulate within collapsed Golgi bodies (Fig. 4), which were identified by labelling with WGA-TR and anti-Golgin-97 (result not shown).

**Inhibition of SubAB-mediated BiP cleavage**

To determine whether any of the above inhibitors were capable of completely blocking retrograde transport of native SubAB to the ER, cleavage of its substrate BiP was assessed by Western blotting. This is an extremely sensitive measure of arrival of toxin in this compartment, as we have previously shown that less than 1 ng ml\(^{-1}\) exogenous SubAB is sufficient to degrade all of the BiP in Vero cell monolayers within 60 min (Paton et al., 2006). While nocodazole and filipin were capable of altering the distribution of SubAB-OG within Vero cells, neither compound protected BiP from cleavage by the toxin, as evidenced by complete loss of the intact (72-kDa) BiP species, and appearance of its 28-kDa cleavage product (Fig. 6A). Similarly, M\(_{\text{b}}\)CD and genistein treatments were unable to prevent BiP cleavage, indicating SubAB internalization is not caveolae/lipid raft-sensitive. However, incubation at 4°C or at 37°C in the presence of acetate, chlorpromazine (CPZ), PAO or BFA completely blocked SubAB-mediated cleavage of BiP (Fig. 6A). Thus, metabolic activity, formation of membrane-bound vesicles, clathrin-dependent endocytosis and a functional Golgi, respectively, are essential for SubAB-mediated trafficking and cytotoxicity.

**Subtilase cytotoxin trafficking in other cell types**

To determine whether cell type influenced the SubAB trafficking pathway, we examined the capacity of a subset of the above inhibitors to block SubAB-mediated cleavage of BiP in HeLa (human cervical carcinoma) and N2A (murine neuroblastoma) cells (Fig. 6B). PAO and brefeldin-A completely prevented BiP cleavage in both cell types, whereas the combination of genistein and M\(_{\text{b}}\)CD did not. Thus, the trafficking pathway of SubAB to the ER appears to be similar in multiple cell types.

**Cell cycle-dependent internalization**

The differential binding of SubAB, StxB and CtxB by Vero cells (Fig. 1) prompted analysis of uptake of either SubAB-OG, CtxB-AF595 and StxB-TR in relation to the
Fig. 4. Inhibition of trafficking. Vero cells were preincubated for 30 min at 4°C or in media supplemented with 60 mM acetate, 1.5 μg ml⁻¹ nocodazole, 25 μg ml⁻¹ CPZ, 1.25 μM PAO, 1.25 μg ml⁻¹ filipin, 100 μg ml⁻¹ genistein, 5 mM MJCD or 0.5 μg ml⁻¹ BFA, and then exposed to 1 μg ml⁻¹ SubAB-OG, 0.5 μg ml⁻¹ CtxB-AF594, or 0.5 μg ml⁻¹ StxB-TR for 3 h, while maintaining inhibitory conditions. Samples were viewed by laser confocal microscopy (60 x oil objective). Scale bars = 25 μm.
cell cycle, by immuno-labelling cell cycle phase markers (Fig. 7A). Examination of multiple fields indicated that most (63%) cells in G1 phase (indicated by nuclear Cyclin E (Ohtsubo et al., 1995)) were capable of internalizing SubAB-OG, as were cells in early S phase (identified by limited BrdU incorporation). These cells constituted 30.5% of all S phase cells. The remaining 69.5% that were further into S phase (indicated by high BrdU labelling) exhibited negligible SubAB-OG uptake, as did cells expressing cytoplasmic Cyclin B1 (G2 phase (Takizawa and Morgan, 2000)). Mitotic chromosome configurations were labelled with anti-Phospho-Histone H3 to identify cells traversing M phase. These cells also demonstrated limited SubAB-OG internalization. In contrast, CtxB-AF594 was taken up by virtually all cells in S through M phases and by approximately 68% of cells in G1 (Fig. 7B), while uptake of StxB-TR occurred preferentially in G1 (57% of cells labelled) (Fig. 7C).

Discussion

Subtilase cytotoxin is the prototype of a distinct family of bacterial AB$_5$ cytotoxins, differing from the other AB$_5$ families in terms of its A subunit enzymatic activity (serine protease for SubAB, vs. RNA-N-glycosidase for Stx, and ADP-ribosylase for Ctx, the related E. coli labile enterotoxins and pertussis toxin). It is also unique in that its only known substrate (BiP) is not located in the cytosol, but rather is a resident ER chaperone. Additional differences exist between SubAB and either Ctx or Stx in terms of target cell receptor specificity, illustrated in the present study by the differential binding of labelled toxins to Vero cells, and differential effects of the cell-cycle on display of the respective receptors. These differences raised the possibility of distinct intracellular trafficking routes. Indeed, a recent study (Morinaga et al., 2007) suggested that SubAB may be translocated to the cytoplasm from an early endosomal compartment, because SubAB colocalized with EEA1, an early endosome marker, but not β-COP, a Golgi marker. Given the subcellular location of the only known substrate of SubAB, this seemed counter-intuitive. Accordingly, in the present study, we have investigated uptake and intracellular trafficking of SubAB in detail, in comparison with CtxB and StxB.

Cell surface accumulation of the toxins at 4°C, or in the presence of acetate, demonstrates that all are actively internalized by membrane-bound vesicles. However, incomplete colocalization (~65% overlap) of SubAB with either CtxB or StxB in cells that internalized both toxins (Fig. 1), suggested that subsequent transport occurs via a retrograde pathway that is similar, but not identical. In particular, the distinct colocalized and non-colocalized puncta demonstrate differences in endocytic mechanisms exploited. Unlike CtxB and StxB, endocytosis of SubAB was totally inhibited in the presence of the clathrin inhibitor PAO. PAO caused surface accumulation of SubAB-OG, and also completely prevented cleavage of BiP in SubAB-treated Vero cells. Thus, not even traces of toxin were internalized and transported to the ER. A similar effect was also seen using CPZ, another clathrin inhibitor, although this also had general cytotoxic effects on Vero cells. In contrast, SubAB was less susceptible than CtxB or StxB to inhibition of trafficking by genistein and/or MβCD, which deplete cholesterol/caveolae. Neither of these inhibitors had a significant impact on trafficking of SubAB-OG, either alone or in combination, nor could these compounds prevent complete cleavage of BiP in

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Fig. 7. Cell cycle-dependent internalization. Vero cells were exposed to 1 μg ml⁻¹ SubAB-OG (A), 0.5 μg ml⁻¹ CtxB-AF594 (B) or 0.5 μg ml⁻¹ StxB-TR (C) for 30 min For S phase labelling, medium was supplemented with 20 μg ml⁻¹ BrdU. DNA was denatured using HCl and neutralized with Borax buffer. Cells were formalin fixed and permeabilized with Triton X-100, and reacted with anti-BrdU, which was detected with anti-mouse-AF488, or anti-mouse-AF594, as appropriate. Nuclear Cyclin E and cytosolic Cyclin B1 indicate cells traversing G1 and G2 phase, respectively, while chromosome configurations of cells in M phase are identified with anti-PH3. Anti-Cyclin E, -B1 and -PH3 were detected with anti-rabbit-AF488, or anti-rabbit-AF594, as appropriate. Scale bars = 25 μm.
cells treated with active toxin. The significant fluorescence colocalization of SubAB-OG with Tf-TR at early time points, and negligible colocalization of SubAB-TR with Caveolin-1-EGFP are both also consistent with the above findings. In contrast, MJ3CD caused marked surface accumulation of CtxB-AF594, while both MJ3CD and genistein affected targeting of StxB-TR to the Golgi. These findings imply that there are clear differences in the extent to which internalization and subcellular trafficking of the three AB5 toxins are dependent upon association with lipid rafts.

Despite the variation of transport routes from the plasma membrane, all three toxins converged at a juxtanuclear region, identified as the TGN/Golgi by labelling with WGA-TR and anti-Golgin-97. Although nocodazole fragments the Golgi apparatus, it does not disrupt its function, and previous studies have shown it has no effect on Golgi transport of vesicular stomatitis virus G protein (Rogalski and Singer, 1984). Accordingly, nocodazole did not abrogate SubAB transport or toxin-mediated cleavage of BiP. Moreover, collapse of the Golgi, TGN and possibly recycling endosomes by treatment with BFA caused accumulation of all three labelled toxin species in that compartment, and importantly, completely prevented cleavage of BiP in SubAB-treated cells. Unlike Ctx and Stx, whose catalytic A subunits must exit the ER to access their cytosolic targets, SubAB-OG became associated with its intracellular substrate BiP in the ER lumen, presumably terminating its intracellular journey in that compartment. However, to address the possibility of BiP being an imprecise marker of the ER due to its role as a chaperone, ER-Tracker Red was also used. While SubAB does not contain an ER-targeting KDEL motif like those found in Ctx and related heat-labile enterotoxins (Lencer and Tsai, 2003), its localization to the ER was nevertheless confirmed. The studies involving colocalization of SubAB-OG with subcellular markers showed that trafficking occurred rapidly, with substantial colocalization with Tf-TR, a clathrin marker, within 5 min and subsequent colocalization with Golgi and ER markers within 30 min of addition of toxin. This is consistent with our previous report that cleavage of BiP could be detected in Vero cells within 20 min (Paton et al., 2006). Our data provide robust support for the conclusion that SubAB undergoes clathrin-dependent retrograde transport, via early endosomes and the Golgi network, to the ER. This disagrees with the recent findings of Morinaga et al. (2007) referred to above. The reason for this discordance is unknown. Morinaga et al. used a slightly different procedure for fluorescent labelling of SubAB, which may have interfered with trafficking. In the present study we used an optimal dye:protein molar ratio of 8:1, which had no effect on trafficking or specific cytotoxicity of the labelled product. However, at higher ratios, trafficking of labelled toxin was almost completely inhibited (result not shown).

An interesting observation in the present study was the marked heterogeneity in terms of toxin uptake by individual cells in Vero monolayers exposed to SubAB-OG and either CtxB-AF594 or StxB-TR. This clearly demonstrates that the three toxins engage distinct receptors. For CtxB and StxB, the preferred receptors are the glycolipids GM1 and Gb3 respectively (Merritt and Hol, 1995). The precise identity of the receptor for SubB is uncertain, although Yahiro et al. (2006) have reported that it is capable of binding α2β1 integrin. In preliminary studies, we have found that SubAB also binds to several components in ganglioside and glycosphingolipid extracts of Vero cells separated by thin-layer chromatography. However, the precise identity of the preferred receptor is yet to be determined (D. Chong, unpubl. obs.). Apart from influencing early trafficking events, the differential target specificity may also influence signal transduction events that may initiate from B subunit–receptor interactions.

Differential internalization of SubAB-OG by Vero cells in a non-synchronous cell population led to the hypothesis that its uptake was influenced by the cell cycle phase, as previously reported for Ctx and Stx in Vero cells (Pudymaitis and Lingwood, 1992: Majoul et al., 2002). Immunolabelling of cell cycle phase markers revealed that Vero cells traversing G1 and early S phases internalized SubAB-OG to a greater extent than those migrating through the remainder of the cycle. In contrast, StxB-TR was taken up predominantly in G1, while CtxB-AF594 uptake occurred throughout the cell cycle except for some cells in G1. Curiously, previous studies on cell cycle-dependent uptake of Stx and Ctx conflict with the above findings, as well as with each other. Pudymaitis and Lingwood (1992) report maximal sensitivity to Stx during early S phase, while Majoul et al. (2002) report that this occurs during G2 phase and mitosis for Stx, and G1 phase for Ctx. It should be remembered, however, that although clear cell cycle-dependent differences in gross uptake of SubAB-OG were observed by fluorescence microscopy, there was low-level baseline labelling of all cells in non-synchronized cultures. Furthermore, the specific enzymatic activity of SubAB is such that this baseline level of toxin uptake is sufficient to cause complete degradation of BiP in Vero monolayers.

Bacterial protein toxins have proven to be useful as tools in cell biology, providing insight into cellular processes and subcellular transport routes. In this study, we demonstrate the internalization and retrograde trafficking of SubAB to its ER-resident target BiP, by an exclusively clathrin-dependent pathway. At present, the reason for the inability of SubAB to engage alternative transport routes from the plasma membrane to the ER such as those exploited by both Ctx and Stx is unknown. However, a key feature may relate to the relative capacity of toxin–
receptor complexes to associate with lipid rafts. Elucidation of the precise receptor specificity of SubAB will clearly contribute to our understanding of this process. The apparently absolute specificity of SubAB for BiP (Paton et al., 2006), and its rapid retrograde transport to the ER compartment, makes SubAB a powerful tool for examining the role of BiP in important cellular functions. These include mediating correct folding of nascent secretory proteins, regulating ER stress signalling, and targeting terminally misfolded proteins to the Sec61 apparatus for degradation by the proteasome (Gething, 1999; Henderson et al., 2004). It is now believed that the catalytic subunits of Stx, Ctx and ricin are retro-translocated from the ER lumen into the cytosol by subversion of the Sec61 pathway, thereby enabling them to engage their respective substrates (Lencer and Tsai, 2003). Furthermore, at least for StxA, this is believed to occur following interaction with BiP and another chaperone HEDJ/ERdj3 (Yu and Haslam, 2005). This latter finding is of particular interest given the fact that to date, SubAB has only been found to interact with BiP and another chaperone HEDJ/ERdj3 (Yu and Haslam, 2005). This raises the possibility that cleavage of BiP by SubAB may directly modulate entry of StxA into the cytosol, and hence the in vivo consequences of Stx intoxication in patients infected with a bacterial strain producing both toxins.

**Experimental procedures**

**Purification and fluorescent labelling of toxins**

Subtilase cytotoxin and SubA272B used for fluorochrome labelling were purified as previously described (Paton et al., 2004; Talbot et al., 2005) and dialysed against PBS. SubAB and SubA272B were labelled with FluoReporter OG or TR Protein Labeling Kits (Molecular Probes), according to the manufacturer’s instructions. Fluorescent labelling had no effect on specific cytotoxicity of SubAB; this was assessed by titration of native SubAB, SubAB-OG and SubAB-TR on Vero cell monolayers in 96-well plates, and examination for cytopathic effect after 3 days incubation, as described previously (Paton et al., 2004). Purified StxB (a gift from Dr Anne V. Kane, Tufts New England Medical Center) was labelled with the FluoReporter TR Protein Labeling Kit. CtxB-AF594 was purchased from Molecular Probes.

**Subcellular markers**

Dextran-TR, Tf-TR, WGA-TR, anti-Human Golgin-97 mouse mAb (CDF4) (Molecular Probes) and anti-BiP (C-20; Santa Cruz Biotech, CA) were used to label endosomes, clathrin-coated vesicles, TGN, Golgi bodies and the SubA target respectively (Mills and Finlay, 1994; Verma et al., 2000; Khine et al., 2004; Paton et al., 2006). LysoTracker DND-99, MitoTracker CMXRos and ER-Tracker Red (Molecular Probes) were used to label lysosomes, mitochondria and the ER respectively. AF594-conjugated donkey anti-mouse IgG was obtained from Molecular Probes.

**Tissue culture**

Vero cells were grown in DMEM (Gibco Life Technologies) with 5% FCS, 100 U ml⁻¹ penicillin G and 100 μg ml⁻¹ streptomycin sulfate at 37°C in 5% CO₂.

**Fluorescence colocalization**

Vero cells grown on coverslips in a 24-well tray were exposed to 1 μg ml⁻¹ SubAB or SubA272B labelled with either OG or TR in tissue culture medium at 37°C. Unbound toxin was washed off with PBS after 30 min and coverslips were subsequently incubated in growth medium for the indicated time period, whereupon the cells were fixed with formalin/PBS. After washing with PBS and H₂O, samples were mounted in ProLong Gold Anti-fade (Molecular Probes) and cured for 24 h.

Vero cells exposed to SubAB-OG were coincubated with 0.5 μg ml⁻¹ StxB-TR, CtxB-AF594, 50 μg ml⁻¹ Dextran-TR, or 5 μg ml⁻¹ WGA-TR for the first 30 min of the desired time period. Medium was supplemented with 100 nM LysoTracker DND-99, 400 nM MitoTracker CMXRos, or 1 μM ER-Tracker Red for 30 min before fixation, as required.

**Immunofluorescence colocalization**

Vero cells exposed to SubAB-OG were fixed as described earlier, then permeabilized with 0.1% (v/v) Triton X-100/PBS, washed with PBS and blocked with 20% (v/v) FCS/PBS at 37°C for 1 h. Golgi and BiP were labelled with anti-Golgin-97 and anti-BiP respectively. Primary antibodies were detected with goat anti-mouse IgG-AF594 or donkey anti-goat IgG-AF594 respectively.

**Transfection of cells**

Vero cells were stably transfected with pCaveolin-1-EGFP (Eyre et al., 2007) using Lipofectamine 2000 (Invitrogen). Transfectants were selected with media containing 800 μg ml⁻¹ geneticin (Invitrogen) for two weeks and then maintained in 250 μg ml⁻¹ geneticin. Co-localization experiments were performed using SubAB-TR, as described above.

**Inhibition of trafficking and cytotoxicity**

Vero cells grown on coverslips were preincubated for 30 min at 4°C or at 37°C in media supplemented with 60 mM acetate (pH ~5), 1.5 μg ml⁻¹ nocardazole, 25 μg ml⁻¹ CPZ, 1.25 μM PAO, 1.25 μg ml⁻¹ filipin, 100 μg ml⁻¹ genistein, 5 mM MgCD or 0.5 μg ml⁻¹ BFA, and then exposed to 1 μg ml⁻¹ SubAB-OG for 3 h while maintaining inhibiting conditions, and then formalin fixed. For inhibition of SubAB-mediated cytotoxicity, unfixed Vero cell lysates from the above experiments were separated by SDS-PAGE and transferred onto nitrocellulose, as described previously (Laemmli, 1970; Towbin et al., 1979). Filters were probed with polyclonal goat anti-BiP, followed by rabbit anti-goat IgG-alkaline phosphatase conjugate (Bio-Rad Laboratories, CA). Labelled bands were visualized using nitro-blue tetrazolium/X-phosphate substrate (Roche Molecular Diagnostics, Germany). The Benchmark Prestained Protein Ladder (Invitrogen) was used as a size marker.
Cell cycle phase identification

Cells in G1 and G2 phases were identified by their subcellular distribution of Cyclin E and B1 respectively. Polyclonal rabbit anti-Cyclin E (H-232) and anti-Cyclin B1 (M-20; Santa Cruz) were gifts from Josephine White (University of Adelaide). Mitotic cells were identified on the basis of chromosomal configuration following immuno-labelling with monoclonal murine anti-Phospho-Histone H3 (PH3) (6G3; Cell Signaling Technology, MA). BrdU incorporation was used to identify cells traversing S phase. Cells were grown in media supplemented with 20 μM BrdU for 30 min, formalin fixed and rinsed twice in wash buffer (0.1% [v/v] Triton X-100/PBS). DNA was denatured for 30 min with 2 M HCl in wash buffer and neutralized with 0.1 M sodium tetraborate 10-hydrate. Samples were blocked with 20% FCS in wash buffer, and incubated with monoclonal murine anti-BrdU (ZBU30; Invitrogen). For detection, samples were incubated with the appropriate AF594-conjugated secondary antibody, while SubAB-OG was resuscitated with AF488-conjugated goat anti-FITC/OG, as required.

Image acquisition and analysis

All samples were viewed using a 60 ¥ 1.4 NA oil objective in an Olympus IMT-2 inverted microscope coupled to a Bio-Rad MRC-600 dual-laser confocal microscope. Excitation wavelengths were obtained from a Krypton/Argon-mixed gas laser (lines at 488 nm and 568 nm for OG and red fluorochromes respectively) with filter sets for OG and TR supplied by the manufacturer. The confocal aperture was set one-third open and 8-bit images were captured using CoMOS v7.1 (Bio-Rad). Images were coloured with filter sets for OG and TR supplied by the manufacturer. Image acquisition and analysis were performed essentially as described by Nichols et al. (2001) where ImageJ v1.38a (National Institutes of Health) was used to perform operations on binary images.

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