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The trans-Golgi SNARE syntaxin 6 is recruited to the chlamydial inclusion membrane

Elizabeth R. Moore,† David J. Mead, Cheryl A. Dooley, Janet Sager and Ted Hackstadt

Correspondence
Ted Hackstadt
thackstadt@niaid.nih.gov

Host-Parasite Interactions Section, Laboratory of Intracellular Parasites, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratories, 903 South 4th Street, Hamilton, MT 59840, USA

Chlamydia trachomatis is an obligate intracellular pathogen that replicates within a parasitophorous vacuole termed an inclusion. The chlamydial inclusion is isolated from the endocytic pathway but fusogenic with Golgi-derived exocytic vesicles containing sphingomyelin and cholesterol. Sphingolipids are incorporated into the chlamydial cell wall and are considered essential for chlamydial development and viability. The mechanisms by which chlamydiae obtain eukaryotic lipids are poorly understood but require chlamydial protein synthesis and presumably modification of the inclusion membrane to initiate this interaction. A polarized cell model of chlamydial infection has demonstrated that chlamydiae preferentially intercept basolaterally directed, sphingomyelin-containing exocytic vesicles. Here we examine the localization and potential function of trans-Golgi and/or basolaterally associated soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins in chlamydia-infected cells. The trans-Golgi SNARE protein syntaxin 6 is recruited to the chlamydial inclusion in a manner that requires chlamydial protein synthesis and is conserved among all chlamydial species examined. The localization of syntaxin 6 to the chlamydial inclusion requires a tyrosine motif or plasma membrane retrieval signal (YGRL). Thus in addition to expression of at least two inclusion membrane proteins that contain SNARE-like motifs, chlamydiae also actively recruit eukaryotic SNARE-family proteins.

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INTRODUCTION

Chlamydiae are significant human pathogens responsible for a number of distinct diseases. *Chlamydia trachomatis* comprises 15 serologically defined variants or serovars associated with diverse disease states including endemic blinding trachoma, sexually transmitted diseases, and a more invasive granulomatous disease, lymphogranuloma venereum (Schachter, 1999). *Chlamydia psittaci* causes zoonotic diseases that occasionally are transmitted to humans. *Chlamydia pneumoniae* contributes to the two to five million cases of respiratory pneumonia per year, although the actual incidence of *C. pneumoniae*-induced disease is unknown (Schachter, 1999).

Chlamydiae have evolved a unique biphasic developmental cycle. The infectious, metabolically dormant form, termed the elementary body (EB), is endocytosed by the host cell and remains within a vesicle termed the inclusion, where it differentiates into a metabolically active but non-infectious reticulate body. The inclusion membrane grows to accommodate the increasing number of organisms, while allowing the organisms to acquire essential amino acids, nucleotides and lipids from the host cell (Hackstadt *et al.*, 1995; Hatch, 1975a, b; McClarty, 1994; Moulder, 1991; Wylie *et al.*, 1997). A fundamental question of chlamydial biology relates to the mechanisms that allow the inclusion to create a unique intracellular organelle permitting survival and replication of the parasite.

Upon infection, the nascent inclusion membrane surrounding the infectious EB is plasma membrane derived, but within a few hours, chlamydial type III secreted proteins modify the inclusion membrane (Fields *et al.*, 2003; Rockey *et al.*, 1995, 2002; Shaw *et al.*, 2000). These modifications are evidenced by the initiation of a number of interactions with the host cell, including dynein-dependent trafficking to the microtubule-organizing centre (Clausen *et al.*, 1997; Grieshaber *et al.*, 2003), and separation of the inclusion from the classical endosomal pathway, including restricted fusion with lysosomes (Al-Younes *et al.*, 1999; Fields & Hackstadt, 2002; Hackstadt,

†Present address: Division of Basic Biomedical Sciences in the School of Medicine, The University of South Dakota, 414 East Clark Street, Vermillion, SD 57069-2390, USA.

Abbreviations: EB, elementary body; FBS, fetal bovine serum; PMRS, plasma membrane retrieval signal; SNARE, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor.

1999; Taraska *et al.*, 1996; van Ooij *et al.*, 1997; Wyrick, 2000), and fusion with Golgi-derived vesicles delivering sphingomyelin and cholesterol to the developing chlamydiae (Carabeo *et al.*, 2003; Hackstadt *et al.*, 1996; Scidmore *et al.*, 1996b).

The properties of lipid acquisition suggest that this trafficking is vesicular in nature (Hackstadt, 1999, Carabeo *et al.*, 2003; Hackstadt *et al.*, 1996; Scidmore *et al.*, 1996b). The specificity of this trafficking only to the chlamydial inclusion (Heinzen *et al.*, 1996), a requirement for chlamydial modification of the inclusion membrane (Scidmore *et al.*, 1996b), and the lack of disruption of normal Golgi processing and export of protein (Scidmore *et al.*, 1996a) suggest a unique trafficking pathway. The acquisition of sphingomyelin, but not glucosylceramide, by chlamydiae further implies specificity of this lipid-trafficking pathway (Moore *et al.*, 2008). Development of a polarized epithelial cell model of chlamydial infection demonstrated that in chlamydia-infected polarized cells, the sphingomyelin retained by the chlamydiae is derived predominantly from the basolateral trafficking pathway, indicating that the chlamydial inclusion preferentially intercepts Golgi-derived, basolaterally targeted exocytic vesicles (Moore *et al.*, 2008). This finding has led us to focus on proteins that govern fusion along basolateral trafficking pathways. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins constitute the predominant mechanism of membrane fusion (Parlati *et al.*, 2002). In this study we examine syntaxins, a family of SNARE proteins, which are associated with trans-Golgi and basolaterally directed membrane fusion events. We demonstrate a specific interaction between syntaxin 6, a trans-Golgi SNARE, and the chlamydial inclusion membrane.

METHODS

Organisms and cell culture. HeLa 229 cells [American Type Culture Collection (ATCC; Manassas, VA; CCL-2.1)], cultivated in RPMI 1640 (Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS) (Hyclone) and 10 μg gentamicin ml^{-1} (Gibco-BRL), were used to propagate *Chlamydia trachomatis* serovar L2 (LGV 434), *C. muridarum* (MoPn/Weiss strain), *C. pneumoniae* (AR-39) and *C. psittaci* (*caviae*) GPIC (HC/BW). Infectious EBs were purified from HeLa cells using a Renografin (Braco Diagnostics) gradient, as described by Caldwell *et al.* (1981). Chlamydial titres were determined as described by Furness *et al.* (1960), by utilizing indirect immunofluorescence with a polyclonal rabbit anti-*C. trachomatis* L2 EB, followed by an anti-rabbit Alexa Fluor-conjugated secondary antibody (Molecular Probes). Multiplicities of infection (m.o.i.) for all experiments are based on inclusion-forming units (i.f.u.) determined in HeLa cells. *Coxiella burnetii* Nine Mile phase II was propagated and purified from Vero cells (ATCC; CCL-81) as previously described (Hackstadt *et al.*, 1992).

HeLa and C2BBe1 cell lines were cultured at 37 °C in 5 % CO_2. C2BBe1 (ATCC CRL-2102) cells were cultivated in DMEM + 2 mM GlutaMax (Invitrogen) supplemented with 10 % FBS, 4 mM L-glutamine, 0.01 mg human transferrin ml^{-1} (Invitrogen) and 10 μg gentamicin ml^{-1}. All eukaryotic cells were passaged based on ATCC-suggested protocols using a 0.25 % trypsin, 0.53 mM EDTA solution (ATCC).

Examination of localization of syntaxin 6 to the chlamydial inclusion

Endogenous syntaxins. To examine localization of endogenous syntaxins to the chlamydial inclusion, C2BBe1 cells were seeded onto glass coverslips in 24-well plates, 48 h prior to infection with *C. trachomatis* L2. At 18 h post-infection, cells were fixed in absolute ethanol at −20 °C for 30 min. Samples were then processed for indirect immunofluorescence using rabbit anti-IncG (inclusion membrane protein), mouse anti-syntaxin 4 (BD Biosciences), mouse anti-syntaxin 16 (Synaptic Systems) or mouse anti-syntaxin 6 (BD Biosciences). All secondary antibodies were conjugated to Dylight Fluors and obtained from Jackson ImmunoResearch Laboratories. Coverslips were mounted to slides using ProLong Gold antifade reagent (Invitrogen). Samples were visualized with an LSM 510 Laser Module Zeiss Axiovert 200M confocal microscope (Carl Zeiss MicroImaging).

eGFP-syntaxin 6. To examine the localization of eGFP-syntaxin 6 (kindly proved by Jeffrey Pessin, Albert Einstein College of Medicine, Bronx, NY, USA) (Watson & Pessin, 2000) to chlamydial inclusions, C2BBe1 cells were diluted and plated onto glass coverslips in 24-well plates the day before the transfection. DNA was diluted to 250 ng per 100 μl of Optimem (Invitrogen), and the PLUS and Lipofectimine-LTX reagents (Invitrogen) were used according to the manufacturer's protocol. Cells were incubated with the DNA–lipid complexes for a minimum of 4 h prior to recovery in culture medium. Cells were then infected with either *C. trachomatis* L2, *C. muridarum* or *C. caviae* for an additional 18 h prior to fixation in 3 % paraformaldehyde, permeabilized with 0.1 % Triton X-100 and 0.5 % SDS in PBS, and processed for indirect immunofluorescence to detect intracellular bacteria. To examine localization of eGFP-syntaxin 6 to *C. pneumoniae* or *Coxiella burnetii* Nine Mile Phase II, cells were infected for 36–72 h prior to transfection with the eGFP-syntaxin 6 construct. An antibody made in rabbits against whole paraformaldehyde-fixed *C. trachomatis* serovar L2 or *C. caviae* EBs was used to detect *C. trachomatis* serovar L2 and *C. muridarum*, or *C. caviae*, respectively. Detection of *C. burnetii* was achieved using an antibody against whole paraformaldehyde-fixed organisms raised in rabbits. A mouse monoclonal antibody raised against *C. pneumoniae* was kindly provided by Harlan Caldwell (NIAID, Rocky Mountain Laboratories, Hamilton, MT, USA).

3XFLAG-syntaxin 6 wild-type and mutants. To examine which domain of syntaxin 6 is involved in localizing the protein to the chlamydial inclusion, eGFP-syntaxin 6 (Watson & Pessin, 2000) was used as a template to make the following syntaxin 6 deletion constructs: ΔH1 (helical domain, encoding amino acids 47–71), ΔH2 (helical/SNARE domain, encoding amino acids 166–225) and ΔYGRL (tyrosine motif encoding amino acids 140–143). The GeneTailor Site-Directed Mutagenesis System (Invitrogen) was used in the production of all constructs, with the primers listed in Table 1. To complete the construction of the ΔH1 and ΔH2 syntaxin 6 mutants, PCR products were digested with *Hin*dIII (New England Biolabs), followed by ligation with T4 DNA ligase (New England Biolabs) and transformed into One-Shot MAX Efficiency DH5α-T1R (Invitrogen). All deletion constructs and wild-type syntaxin 6 were subcloned into p3XFLAG-CMV 7.1 expression vector (Sigma Aldrich) using Phusion High Fidelity Polymerase (New England Biolabs) and primers 7 and 8 (Table 1). All mutations were confirmed by sequencing (SeqWright). 3XFLAG-syntaxin 6 constructs were transformed into C2BBe1 cells as described above.

Table 1. Primers used in cloning syntaxin 6 (stx6)

Primer	Sequence	Purpose
1	for 5′-GCTGGAGTGACGGATCGAGACCGGGAGC-3′	Delete YGRL sequence from pEGFP stx6
2	rev 5′-TCGATCCGTCACTCCAGCATCCCAATTCTGGC-3′	
3	for 5′-GGGGGAAGCTTGGTCCAGTCGATCTCTTCCC-3′	Delete H1 domain from pEGFP stx6
4	rev 5′-GGGGGAAGCTTGAAGCAATCCTAGAAAATTCAACC-3′	
5	for 5′-GGGGGAAGCTTTCTCACATGACCAGTGATCGG-3′	Delete H2 domain from pEGFP stx6
6	rev 5′-GGGGGAAAGCTTAATCAACTGCTGCTGTGCCTG-3′	
7	for 5′-GGGGAATTCAATGTCCATGGAGGACCCC-3′	Clone stx6 constructs into p3XFLAG-CMV 7.1 expression vector
8	rev 5′-TCTAGATCCGGTGGATCCCGGGCCCGCGG-3′	

mCherry-syntaxin 6. Syntaxin 6 was subsequently cloned into mCherry (Clontech) and transfected into HeLa cells as described above.

RESULTS

Co-localization of syntaxin 6 with the chlamydial inclusion

C. trachomatis serovar L2 obtains sphingomyelin from a Golgi-derived basolaterally targeted pathway via an unknown mechanism (Moore *et al.*, 2008). Given the integral role of SNARE proteins in mediating vesicular trafficking, we examined whether syntaxin family members co-localized with the chlamydial inclusion in *C. trachomatis*-infected C2BBe1 cells by indirect immunofluorescence. The trans-Golgi-associated syntaxin 6 was found to associate with the chlamydial inclusion (Fig. 1). Syntaxin 6 displays a distinct ring-like staining pattern around the chlamydial inclusion similar to the inclusion membrane protein IncG. Syntaxin 4, known to control fusion to the basolateral plasma membrane (Low *et al.*, 1996; Teng *et al.*, 2001), and syntaxin 16, a ubiquitious trans-Golgi-associated syntaxin which mediates retrograde endosomal-Golgi transport (Mallard *et al.*, 2002), did not colocalize with the chlamydial inclusion but can be observed on the plasma membrane (Fig. 1a).

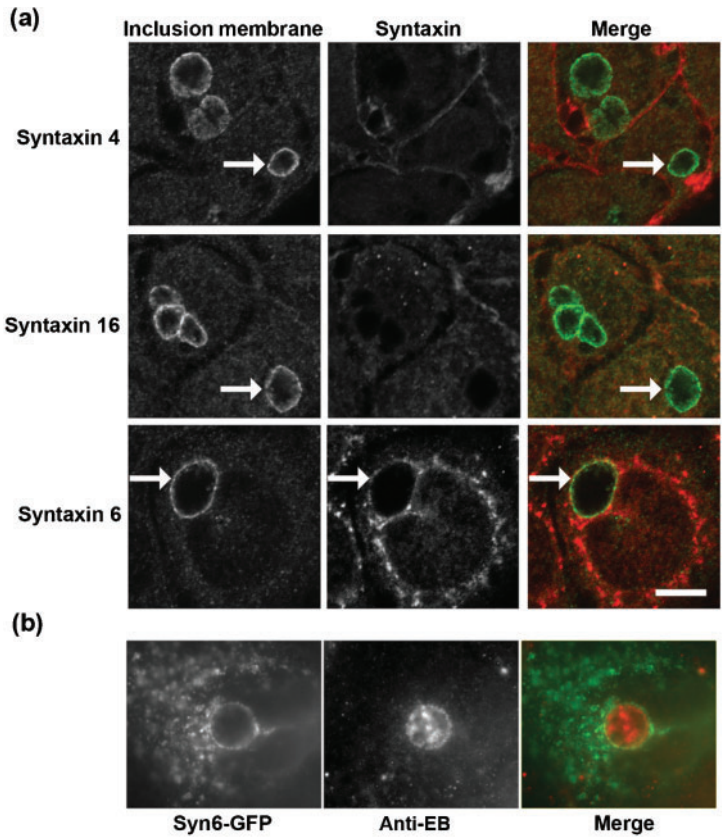

Fig. 1. Localization of endogenous syntaxin proteins to the chlamydial inclusion. (a) C2BBe1 cells were seeded onto glass coverslips for 48 h prior to infection with *C. trachomatis* serovar L2 (m.o.i. 6 : 1) for an additional 18 h. Cells were fixed in absolute ethanol for 30 min at −20 °C and processed essentially as described in Methods. Samples were visualized with an LSM 510 Laser Module Zeiss Axiovert 200M confocal microscope. Arrows indicate chlamydial inclusions. (b) C2BBe1 cells were transfected with eGFP-syntaxin 6 and infected with *C. trachomatis* L2 for 18 h. Cells were fixed with methanol and counterstained with an anti-EB antiserum. Bars, 10 μm.

To confirm this interaction, HeLa cells were transiently transfected with eGFP-syntaxin 6 and infected with *C. trachomatis*. Syntaxin 6 was found to localize to the chlamydial inclusion membrane (Fig. 1b). These findings suggest that syntaxin 6 is specifically recruited to the chlamydial inclusion membrane.

Syntaxin 6 recruitment is conserved among chlamydia species

Because sphingomyelin trafficking to the inclusion is a conserved feature among chlamydial species (Hackstadt *et al.*, 1995; Rockey *et al.*, 1996; Wolf & Hackstadt, 2001), we next examined whether mCherry-syntaxin 6 would colocalize to the inclusion membrane of other *Chlamydia* species. mCherry-syntaxin 6 was recruited to inclusions formed by *C. trachomatis* serovar L2, C. *muridarum*, *C. pneumoniae* and *C. caviae* (Fig. 2). Syntaxin 6 localized to the inclusion membrane of *C. muridarum* and *C. pneumoniae* inclusions in a similar manner as seen for *C. trachomatis* serovar L2 (Fig. 2). Syntaxin 6 clustered amongst the multiple lobed inclusions formed by *C. caviae*; however, the morphology was distinct from the ring-like pattern of syntaxin 6 surrounding the inclusions of chlamydial species that form a single inclusion within the host cell. Syntaxin 6 was not recruited to the parasitophorous vacuole formed by the unrelated intracellular bacterium, *Coxiella burnetii* Nine Mile Phase II (Fig. 2). The interaction of syntaxin 6 with the chlamydial inclusion therefore appears to be chlamydia-specific and is conserved across chlamydial species.

Requirement of chlamydial protein synthesis for syntaxin 6 colocalization

Because sphingomyelin trafficking to the chlamydial inclusion requires chlamydial protein synthesis (Hackstadt *et al.*, 1996; Scidmore *et al.*, 1996b, 2003), we examined whether chlamydial protein synthesis was also required for syntaxin 6 colocalization. C2BBe1 cells were infected with *C. trachomatis* and allowed to develop for 18 h, then treated with chloramphenicol for an additional 24 h (Fig. 3). C2BBe1

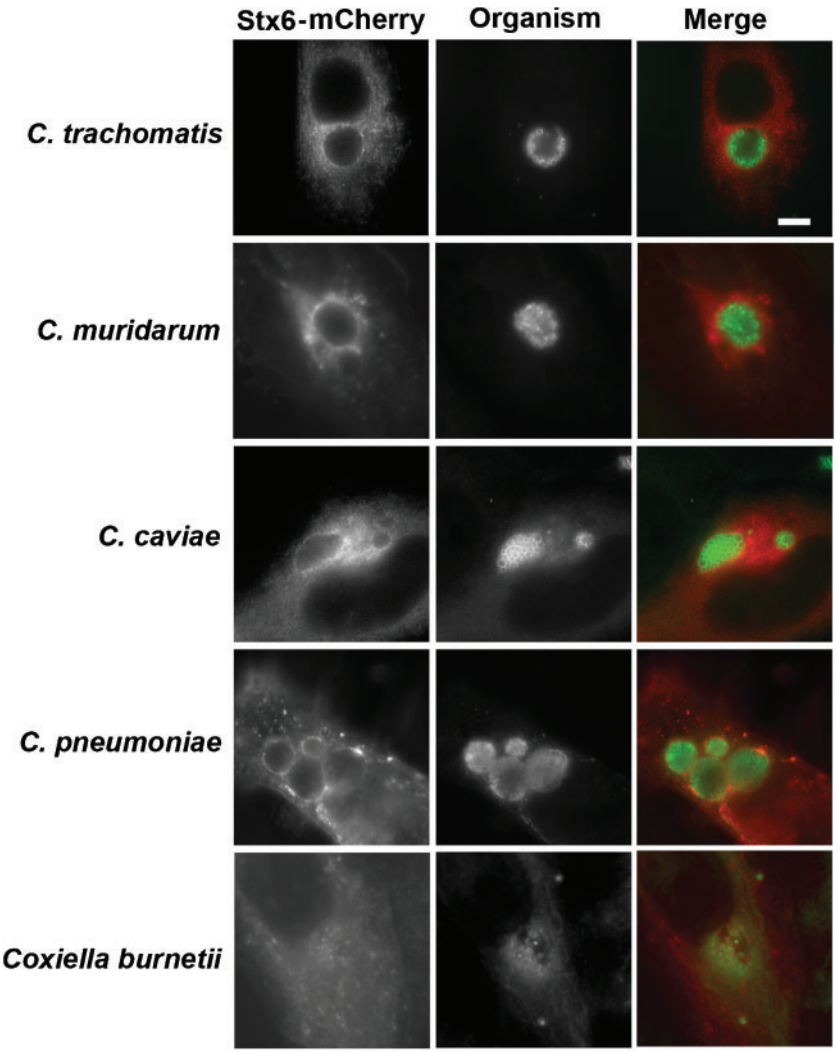

Fig. 2. Localization of syntaxin 6 to the inclusions of multiple chlamydial species. C2BBe1 cells were seeded onto coverslips and transfected with mCherry-syntaxin 6 or with eGFP-syntaxin 6, then infected with *C. trachomatis* serovar L2 (m.o.i. 6 : 1), *C. muridarum* (m.o.i. 0.1 : 1), *C. caviae* (m.o.i. 0.2 : 1), *C. pneumoniae* (m.o.i. 13 : 1) or *Coxiella burnetii* Nile Mile phase II (m.o.i. 50 : 1) as described in Methods. To terminate the infections, cells were fixed in methanol and processed for indirect immunofluorescence to detect the organisms (green). Samples were visualized with an LSM 510 Laser Module Zeiss Axiovert 200M confocal microscope. Bar, 10 μm.

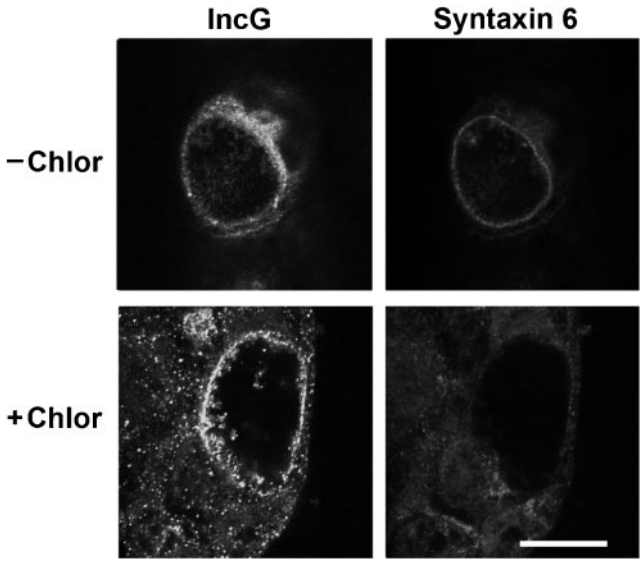

Fig. 3. Chlamydial protein synthesis requirement for syntaxin 6 localization to the inclusion. C2BBe1 cells were seeded onto glass coverslips in 24-well plates 48 h prior to infection with *C. trachomatis* serovar L2 (m.o.i. 9 : 1). After 18 h, cells were either fixed in absolute ethanol (−Chlor) or treated with 200 μg ml^{-1} for an additional 24 h (+Chlor), then fixed in absolute ethanol and processed for indirect immunofluorescence essentially as described in Methods. IncG staining was used to identify the inclusion. Samples were visualized with an LSM 510 Laser Module Zeiss Axiovert 200M confocal microscope. Bar, 10 μm.

cells fixed at 18 h post-infection displayed the characteristic ring-like pattern of syntaxin 6 localization to the inclusion membrane. However, no inclusion-membrane-associated staining of syntaxin 6 was visible in chloramphenicol-treated cells (Fig. 3). These results suggest that syntaxin 6 localization to the inclusion and retention at the inclusion membrane are likely mediated by a chlamydial protein. Treatment of *C. trachomatis*-infected cells at 18 h post-infection with brefeldin A, which collapses the Golgi (Lippincott-Schwartz *et al.*, 1989), or nocodozole, which disrupts microtubules and fragments the Golgi apparatus (Cheung & Terry, 1980; Tassin *et al.*, 1985), did not inhibit syntaxin 6 retention or recruitment to the inclusion membrane (data not shown).

Characterization of the syntaxin 6 domain responsible for localization to the chlamydial inclusion

Syntaxin 6 contains two helical domains (H1 and H2) and a plasma membrane retrieval signal (PMRS) which are required for syntaxin 6 cycling and function in eukaryotic cells (Fig. 4a). The H1 domain resides within the N-terminus of syntaxin 6 and is responsible for protein–protein interactions as characterized by its interaction with α-SNAP (Bock *et al.*, 2001). The H2 domain resides proximal to the C-terminal transmembrane domain and comprises the Q-SNARE activity, which is similar to the SNARE activity of SNAP25 (Bock *et al.*, 2001; Watson & Pessin, 2000). Within the C-terminal portion of syntaxin 6 is a 10 amino acid domain, which is characterized as the PMRS, with residues YGRL being absolutely required for activity (Watson & Pessin, 2000). Because syntaxin 6 is trafficked within vesicles from the Golgi apparatus to the plasma membrane, the YGRL signal sequence is required for the recycling of syntaxin 6 from the plasma membrane to the trans-Golgi region. Deletion of this domain causes an accumulation of syntaxin 6 in the plasma membrane (Watson & Pessin, 2000). Both the H2 and PMRS domains contribute to the trans-Golgi localization of syntaxin 6 . To understand which signalling or protein–protein binding domains facilitate the trafficking of syntaxin 6 to the chlamydial inclusion, we constructed a series of syntaxin 6 mutants from which the H1, H2 or PMRS domains from syntaxin 6 were deleted. Subsequently, C2BBe1 cells were transfected with 3XFLAG wild-type and mutant syntaxin 6 constructs and their subcellular localization relative to *C. trachomatis* serovar L2 inclusions was examined (Fig. 4b). Deletion of either the H1 or H2 domain did not have any appreciable effect on syntaxin 6 localization to the chlamydial inclusion. However, deletion of the PMRS domain resulted in loss of recruitment of syntaxin 6 to the chlamydial inclusion membrane (Fig. 4b). These results suggest that the PMRS may be acting as signal sequence which targets eukaryotic proteins to the chlamydial inclusion, and/or that the chlamydial inclusion membrane may be mimicking the trans-Golgi membrane.

DISCUSSION

Recent studies have indicated that the chlamydial inclusion preferentially intercepts sphingomyelin from a basolaterally directed pathway (Moore *et al.*, 2008). The recognition of a specific pathway targeted by chlamydiae stimulated a search for host proteins that may serve to regulate this pathway. Syntaxin 6 is recruited to the chlamydial inclusion in a process that requires chlamydial protein synthesis and is conserved across chlamydial species. Interestingly, the PMRS signal of syntaxin 6 is required for syntaxin 6 colocalization to the inclusion.

Vesicle fusion in eukaryotic cells is of necessity a highly regulated process designed to maintain the integrity and distinction of intracellular compartments. Specificity in vesicle fusion with target membranes is conferred by integral membrane proteins termed v-SNAREs and t-SNAREs for vesicle- and target-specific soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP) receptors, respectively (Rothman & Wieland, 1996). Binding of t-SNAREs with v-SNAREs on opposing membrane faces causes the vesicles to dock with the acceptor membrane. v-SNAREs and t-SNAREs have more recently been termed Q-SNAREs and R-SNAREs, respectively, based on conserved amino acids within the SNARE protein fusion

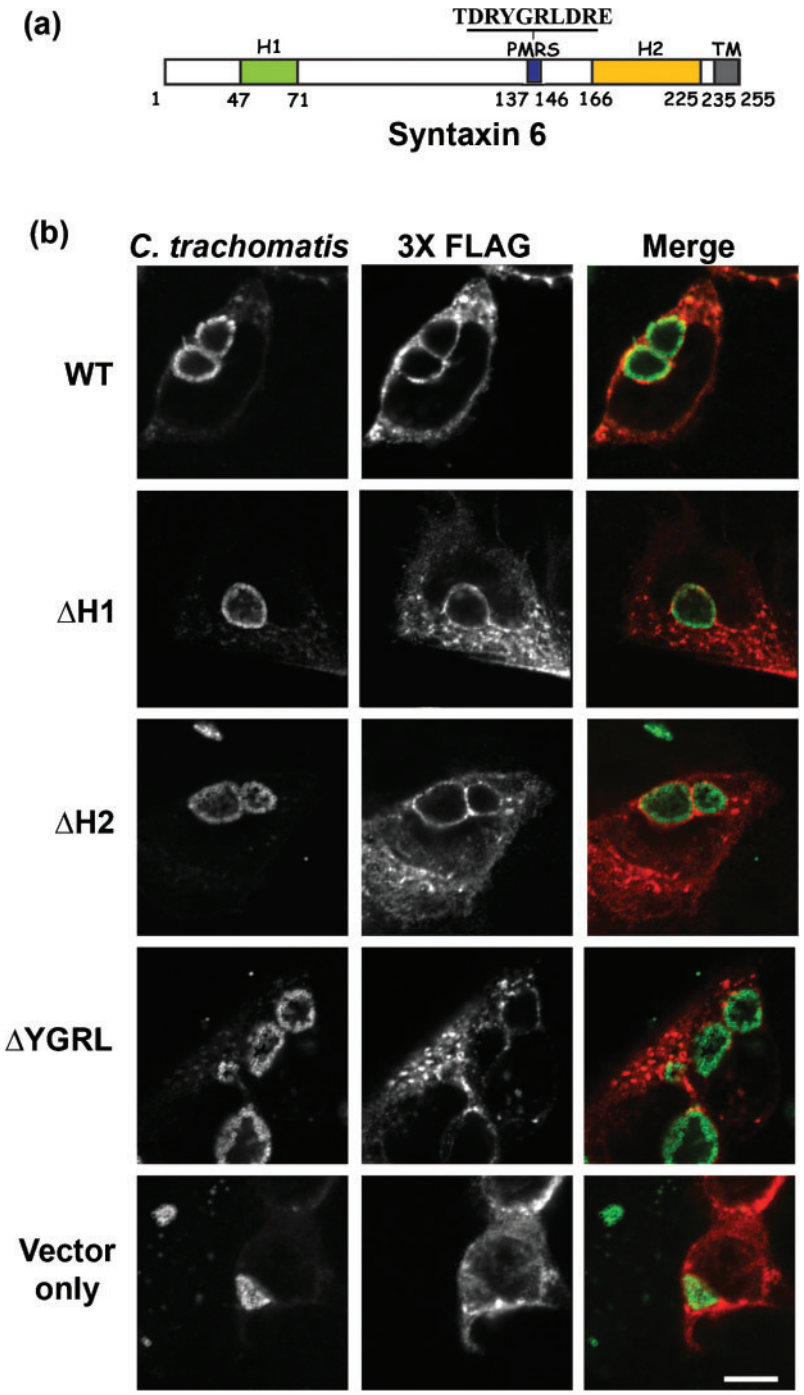

Fig. 4. Identification of the syntaxin 6 protein domain mediating localization to the chlamydial inclusion. (a) Functional domains of syntaxin 6. These include the 24 amino acid H1 domain, the 10 amino acid plasma membrane retrieval signal (PMRS), including the YGRL tyrosine motif and the 59 amino acid H2 domain. Also depicted is the C-terminal transmembrane domain (TM); this domain anchors syntaxin 6 in vesicular membranes (Wendler & Tooze, 2001). (b) Examination of the involvement of syntaxin 6 functional domains in localization to the chlamydial inclusion. C2BBe1 cells were transfected with the indicated 3XFLAG-syntaxin 6 (syn6) constructs, followed by infection with *C. trachomatis* serovar L2 (m.o.i. 4 : 1). At 18 h post-infection, cells were fixed in absolute ethanol and processed for indirect immunofluorescence. Chlamydial inclusions were labelled with an antibody that recognizes the inclusion membrane (IncG), and the syntaxin 6 constructs were detected with an anti-M2 FLAG tag antibody (Sigma-Aldrich). Slides were visualized with an LSM 510 Laser Module Zeiss Axiovert 200M confocal microscope. Bar, 10 μm.

complex. Once the complex is formed, two soluble proteins, *N*-ethylmaleimide-sensitive factor (NSF) and SNAP, bind the complex. Subsequent ATP hydrolysis by NSF promotes actual membrane fusion. Several other host proteins are also involved in regulation of vesicle trafficking. Specific small GTPases of the Rab family are localized to the surface of the various compartments of the endocytic and exocytic pathways where, depending upon the concentration of the GTP-bound state, they positively or negatively regulate the rates of SNARE complex assembly and membrane fusion (Novick & Zerial, 1997; Schimmöller *et al.*, 1998).

Microbial manipulation of host SNARE machinery is an emerging theme in cellular microbiology. *Mycobacterium tuberculosis*-containing vacuoles (MCVs) transiently acquire syntaxin 3, acquire and retain syntaxins 4 and 8, but exclude syntaxin 6 (Fratti *et al.*, 2003; Parlati *et al.*, 2002; Perskvist *et al.*, 2002). Each step of syntaxin acquisition or exclusion marks a critical step in the MCV maturation from a plasma-membrane-derived vacuole. Simlarly, syntaxin 13 appears to play a role in maturation of the *Salmonella*-containing vacuole (Smith *et al.*, 2005). Virulent *Legionella pneumophila* require syntaxins 2, 3 and

4 for proper vacuolar biogenesis and fusion with endoplasmic-reticulum-derived vesicles (Arasaki & Roy, 2010). As shown here, *C. trachomatis* excludes syntaxins 4 and 16, but recruits syntaxin 6, a trans-Golgi SNARE protein. While the SNARE domain of syntaxin 6 is not involved in localizing the protein to the chlamydial inclusion, we hypothesize that the SNARE domain plays an important role at the inclusion membrane and may mediate specific vesicle fusion events. The role(s) of syntaxin 6, however, remain undefined as siRNA depletion did not dramatically diminish inclusion development or trafficking of sphingomyelin to the inclusion (data not shown).

Chlamydiae are known to extensively modify the inclusion membrane very early in infection by the insertion of type III secreted intrinsic membrane proteins collectively known as Incs. The Inc proteins show little similarity to known host proteins but display a predicted, bi-lobed hydrophobic domain approximately 40 amino acids in length. *C. trachomatis* encodes up to 50 Inc proteins (Rockey *et al.*, 2002; Shaw *et al.*, 2000). In addition to recruiting eukaryotic SNARE proteins to the inclusion membrane, Inc proteins may mimic eukaryotic SNAREs. Computer modelling has identified two chlamydial proteins, IncA and CT813, as having SNARE-like domains (Delevoye *et al.*, 2004, 2008). Additionally, *in vitro* studies utilizing reconstituted liposomes with recombinant proteins have found that IncA can bind the SNARE protein vamp 3 *in vitro* (Delevoye *et al.*, 2008). Interestingly, vamp 3 operates along a basolateral trafficking pathway in polarized epithelial cells (Pocard *et al.*, 2007).

C. trachomatis IncA is required for homotypic vesicle fusion of multiple *C. trachomatis* inclusions within the same cell. Other chlamydia species also carry genes annotated as IncA; however, *C. caviae* IncA does not mediate fusion with other inclusions formed by *C. caviae* or other chlamydial species. It is likely that host factors are also required for *C. trachomatis* homotypic vesicle fusion (Delevoye *et al.*, 2008). In addition to promoting fusion of *C. trachomatis* inclusions, IncA has also been proposed to act as an inhibitory SNARE by blocking specific SNARE-mediated membrane fusion events *in vitro* (Paumet *et al.*, 2009). Interestingly, *C. trachomatis* IncA had no inhibitory effect on exocytic complexes examined but was specific for endocytic SNAREs (Paumet *et al.*, 2009). This is particularly relevant since chlamydial inclusions are non-fusogenic with endocytic compartments but are believed to intercept sphingolipids and cholesterol from exocytic vesicles.

Several Rab-family GTPases are also recruited to the chlamydial inclusion membrane but not in patterns common throughout the genus. For example, Rab1, Rab4, Rab11 and Rab14 are recruited to *C. trachomatis, C. muridarum* and *C. pneumoniae* inclusions. Rab 6 is recruited to *C. trachomatis* exclusively, however, and Rab10 is recruited only to *C. muridarum* and *C. pneumoniae* inclusions (Brumell & Scidmore, 2007; Rzomp *et al.*, 2003). The endocytic Rabs, Rab5, Rab7 and Rab9, are excluded from the chlamydial inclusion. Rab4 recruitment to *C. trachomatis* inclusions is mediated by Inc229 (Rzomp *et al.*, 2006) whereas *C. pneumoniae* Cpn585 interacts with Rabs 1, 10 and 11, but not Rab 4 (Cortes *et al.*, 2007). How this combination of Rab proteins associated with different compartments and pathways affects inclusion membrane fusion events remains to be fully defined, although recent studies have implicated a role in mediating the phosphoinositide composition of the inclusion membrane (Moorhead *et al.*, 2010).

Syntaxin 6 is recruited to the chlamydial inclusion membrane protein by a mechanism that requires both an unknown chlamydial protein, presumably one localized to the inclusion membrane, and a plasma membrane retrieval signal on syntaxin 6. SNARE complexes consist of three proteins, which overall contribute three Q-SNARE motifs and one R-SNARE motif (Fasshauer *et al.*, 1998). In a typical SNARE complex, a syntaxin supplies one Q-SNARE motif, a vamp supplies the single R-SNARE motif, and cytosolic SNAP 23 supplies the additional two Q-SNARE motifs (Sutton *et al.*, 1998). SNARE proteins, such as vamps and syntaxins, remain membrane bound whether or not they are found within a complex, and control fusion events within distinct subcellular compartments (Teng *et al.*, 2001). An improved understanding of the chlamydial proteins involved in conferring specificity to the cellular interactions of the chlamydial inclusion is critical to elucidating chlamydial pathogenesis.

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