Cellular Microbiology (2002) **4**(1), 11–18

Cell vacuolization induced by *Helicobacter pylori* VacA cytotoxin does not depend on late endosomal SNAREs†

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Summary

Cellular vacuoles induced by the *Helicobacter pylori* vacuolating cytotoxin VacA originate from late endosomal compartments. Their biogenesis requires the activity of both rab7 GTPase and the ATPase proton pump. The toxin has been suggested to cause an increased luminal osmotic pressure via its anion-specific channel activity localized on late endosomal compartments after endocytosis. Here, we show that the extensive membrane fusion that takes place in the transition from the small late endosomal compartments to the large vacuoles does not depend on soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins. The process of vacuolization leads to disappearance of the large array of internal membranes of late endosomes. We suggest that most of the vacuole-limiting membrane derives from internal membranes.

Introduction

The vacuolating cytotoxin VacA is a major virulence factor of *Helicobacter pylori* (Salama *et al*., 2001), a bacterium that colonizes the stomach mucosa of the majority of the human population and induces gastroduodenal ulcers and stomach cancers in a sizeable proportion of infected patients (Warren and Marshall, 1983; Marshall *et al*., 1985; Parsonnet, 1998; Montecucco and Rappuoli, 2001). This toxin is synthesized as a 140 kDa precursor protein, whose C-terminal domain is removed at the bacterial surface, and the VacA toxin is released into the extracellular medium as a 95 kDa mature protein. VacA spontaneously oligomerizes into inactive holigomers (Cover *et al*., 1994; 1997; Phadnis *et al*., 1994; Schmitt and Haas, 1994; Telford *et al*., 1994; Lupetti *et al*., 1996), which are activated by a short exposure to acid or alkaline pH (de Bernard *et al*., 1995; Yahiro *et al*., 1999). The acid-activated toxin becomes resistant to pepsin degradation (de Bernard *et al*., 1995).

Cytotoxin VacA has a major effect on cells in culture, which develop intracellular vacuoles in the presence of membrane-permeant amines (Leunk *et al*., 1988; Cover *et al*., 1992; Ricci *et al*., 1997). Such cell alteration follows the accumulation of membrane-permeable weak bases, including dyes such as neutral red (Cover *et al*., 1992), because the vacuolar lumen is acidic. This provides a simple and quantitative assay of the internal volume of these compartments.

These vacuoles contain membrane protein markers of late endosomes and lysosomes (Papini *et al*., 1994; Molinari *et al*., 1997) and are capable of incorporating fluid phase markers of extracellular medium (Catrenich and Chestnut, 1992; Cover *et al*., 1992; Papini *et al*., 1994). The vacuolar membrane contains an active vacuolar ATPase proton pump (V-ATPase) (Papini *et al*., 1996), whose inhibition with bafilomycins prevents and reverts vacuole formation (Cover *et al*., 1993; Papini *et al*., 1993). These results indicate that vacuole formation and maintenance depends on the proton pump activity and suggest that intraendosomal accumulation of osmotically active molecules plays a crucial role in cell vacuolization by favouring water uptake and swelling. This hypothesis is strongly supported by the finding that VacA forms voltage-dependent, anion-selective channels in planar lipid membranes and in cells, essential to induce the transformation of late endosomal compartments into vacuoles (Szabò *et al*., 1999; Tombola *et al*., 1999).

The formation of large vacuoles (2–5 μm) from small compartments such as late endosomes and lysosomes has to involve membrane fusion events to account for such a large increase in size. Accordingly, vacuole formation was found to be strictly dependent on the small GTP-binding protein Rab7 (Papini *et al*., 1997), which is involved in membrane trafficking at the level of late endosomes and lysosomes (Feng *et al*., 1995; Meresse *et al*.,

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†This article is dedicated to the memory of Professor Franco Tato.

1995), and in late endosome homotypic fusion (Haas *et al*., 1995).

A wealth of experimental work documents the involvement of soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) proteins, vesicle-associated membrane protein (VAMP), syntaxin and soluble NSF attachment protein (SNAP), and the accessory proteins NSF and α-SNAP, in the various events of membrane fusion taking place during vesicular trafficking within the cell (Rothman, 1994; Jahn and Sudhof, 1999; McNew *et al*., 2000). Many different SNARE proteins have been characterized, and they are responsible for the specificity of the single fusion events. The formation of a very stable four-stranded helical bundle, composed of SNAREs from opposite membranes, is thought to be the central event that drives membrane fusion (Poirier *et al*., 1998; Weber *et al*., 1998). The soluble factors α-SNAP and N-ethylmaleimide (NEM)-sensitive factor (NSF) play the essential role of molecular chaperones to dissociate SNARE protein complexes in such a way that they can be recycled, allowing SNAREs to reform complexes that will drive subsequent rounds of membrane fusion (Sollner *et al*., 1993; Mayer *et al*., 1996). Late endosome fusion has been shown recently to depend on selected isoforms of SNARE proteins (Antonin *et al*., 2000).

To gain further insights into the mechanism of vacuole formation, we have investigated here the possible involvement of SNARE-mediated events of membrane fusion. We have found no evidence for their involvement. On the basis of this result and of a morphological examination of vacuoles, we are proposing here a novel mechanism of vacuole biogenesis from late endosomal compartments, which mainly implicate the large array of intraorganellar membranes.

Results and discussion

In a first set of experiments, we tested the involvement of α-SNAP in VacA-induced vacuolization by examining the effect of a mutant of the cytosolic factor α-SNAP, α-SNAP (L294A), which is reported to block SNARE priming, acting as a dominant-negative effector (Barnard *et al*., 1997). HeLa cells were transfected with a pGEM construct combined with a vaccinia virus infection (Fuerst *et al*., 1986). This approach offers the advantage of a high proportion of transfected cells, making possible a quantitative evaluation of the effect of the mutated protein on VacA-induced vacuolization.

After 14 h of expression, cells were incubated with 100 nM VacA and processed for immunofluorescence. In all fields examined (for an example, see Fig. 1A and B), transfected cells show a clear cytosolic staining for α-SNAP (L294A), but do not develop a vacuolated phenotype. The effect of the cytosolic expression of the dominant-negative α-SNAP (L294A) was evaluated quantitatively by measuring neutral red uptake (NRU), which is known to accumulate in the acidic lumen of the vacuoles (Cover *et al*., 1991). Figure 1C shows that α-SNAP (L294A) completely prevents vacuole formation, whereas control cells transfected with the plasmid pGEM-Myc vacuolate upon exposure to VacA to the same extent as untransfected cells.

However, as endocytosis is SNARE dependent and VacA has to be endocytosed to exert its effect, the

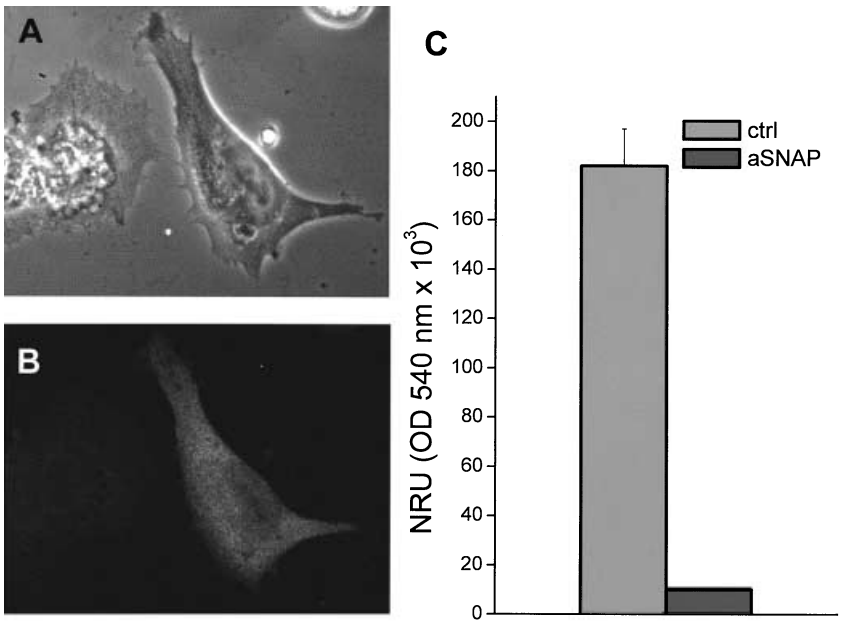

Fig. 1. Effect of α-SNAP(L294A) on vacuolization in VacA intoxicated cells. HeLa cells were transfected with the construct pGEM-Myc-α-SNAP(L294A). After 12 h of expression, cells were incubated for a further 4 h with 100 nM VacA in DMEM, 2% FCS, 5 mM NH_4Cl before being processed for immunofluorescence. Cells were stained to detect transfectants with an anti-Myc tag monoclonal antibody (B). Phase contrast of the same cells is shown in (A). Magnification 500×.
C. Extent of vacuolation induced by VacA on HeLa cells overexpressing mutated α-SNAP, as determined by neutral red uptake. Control (ctrl) corresponds to mock transfected cells. Values are the means of two experiments, and bars represent the range.

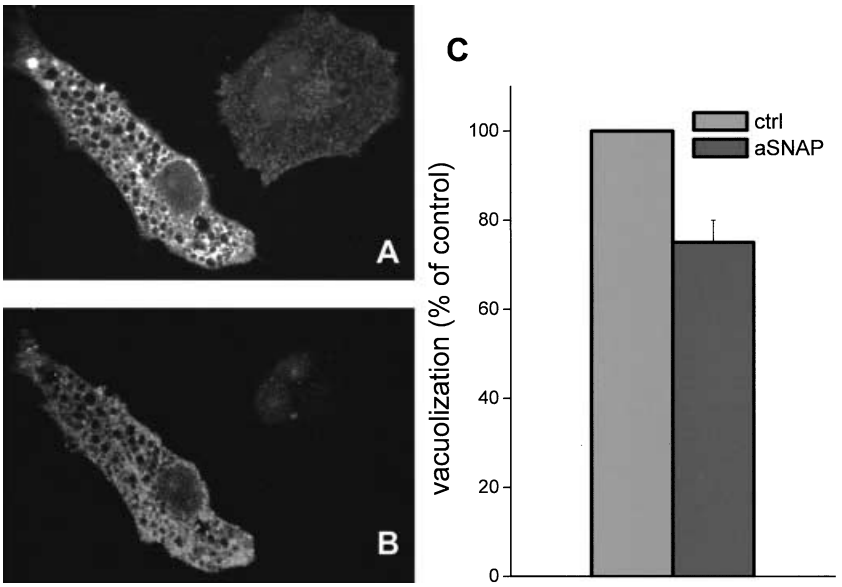

Fig. 2. Effect of α-SNAP(L294A) on vacuolization in VacA overexpressing cells. HeLa cells were co-transfected with pGEM-Myc-α-SNAP(L294A) and pGEMp95. After transfection, cells were incubated for 12 h in DMEM, 2% FCS plus 5 mM NH_4Cl before processing for immunofluorescence. Anti-VacA polyclonal antibody and anti-Myc tag monoclonal antibody were used to stain VacA (A) and α-SNAP (B) in transfected cells. Magnification 500×.
C. Extent of vacuolation in HeLa cells co-transfected with pGEMp95 and pGEM-Myc-α-SNAP(L294A). Control (ctrl) corresponds to cells transfected with pGEMp5 and pGEM-Myc. Values are corrected for the percentage of co-transfection, as estimated by immunostaining, and are the mean of two experiments. Bars represent the range.

inhibitory effect of α-SNAP (L294A) may reflect a block of VacA internalization. Indeed, the overexpression of α-SNAP (L294A) strongly inhibits peroxidase internalization with a reduction ranging between 70% and 90% with respect to mock transfected cells (not shown). In order to clear up this point, we built on previous findings that the cytosolic expression of VacA does induce vacuole formation (de Bernard *et al*., 1997; 1998; Ye *et al*., 1999). We co-transfected cells with pGEM-Myc-α-SNAP (L294A) and pGEM-p95, a plasmid coding for the mature toxin. As shown in Fig. 2A and B, after 12 h, cells expressing both proteins developed vacuoles similarly to control cells (transfected only with pGEM-p95). A quantitative evaluation (Fig. 2C) revealed that the mutant exerts a small inhibitory effect, but these data indicate that a functional SNARE complex is not essential in the vacuolization process, and the strong inhibition of vacuolization observed in transfected cells exposed to the toxin (Fig. 1) has to be attributed to a block of its endocytosis or to inhibition of early endosomal transport.

We then tested the involvement of the protein syntaxin 7, which was shown recently to be directly involved in late stages of the endocytic pathway, either in homotypic fusion between late endosomes or in heterotypic fusion between late endosomes and lysosomes (Mullock *et al*., 2000; Nakamura *et al*., 2000; Ward *et al*., 2000). Our initial approach was based on the observation that recombinant forms of syntaxin 7, lacking the C-terminal transmembrane domain, inhibit homotypic and heterotypic fusion of purified vesicles *in vitro* (Ward *et al*., 2000). The overexpression of the truncated syntaxin 7 in HeLa cells does not inhibit VacA-induced vacuole formation (not shown).

Mullock *et al*. (2000) have shown that anti-syntaxin 7 antibodies inhibit late endosome fusion *in vitro*. We used the same antibodies in a late endosome fusion assay. As depicted in Fig. 3A, we observed a drop of 70% in fusion when the two vesicle populations, loaded with biotinylated horseradish peroxidase (HRP) and avidin, respectively, were preincubated with 60 μg ml^{-1} polyclonal anti-syntaxin 7 antibody for 15 min before proceeding with the fusion. An anti-VAMP 1 antibody used as control in the same experiment did not exert any inhibitory effect (not shown). Based on these results, we tested the effect of microinjected anti-syntaxin 7 antibodies on VacA-induced vacuolization, and the result is illustrated in Fig. 3B and C: microinjected cells, stained with a secondary fluorophore-conjugated antibody, developed a vacuolated phenotype when exposed to the toxin exactly as control cells do. This result indicates further that SNARE-dependent membrane fusion events play little if any role in the formation and growth of vacuoles induced by VacA. These findings account for the fact that we never observed vacuole–vacuole fusion events in prolonged video recordings of cells exposed to VacA (our unpublished results).

If SNARE-dependent fusion of late endosomes is not implicated in vacuole formation and growth, where does the large vacuole-limiting membrane come from? Fig. 4 shows electron micrograph pictures of HeLa cells that had endocytosed BSA–gold to label late endosomal compartments and were then exposed to VacA for different time periods. The vacuoles contain the gold particles, but appear to have lost all the convoluted array of internal membranes, characteristic of the late endosomal

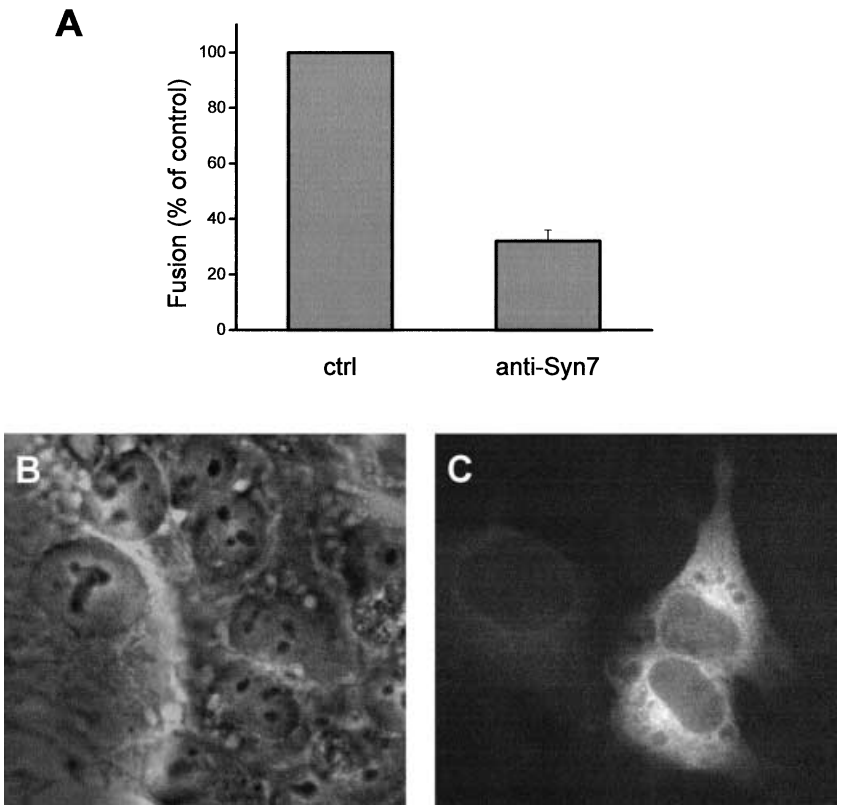

Fig. 3. Role of syntaxin 7 in VacA-induced vacuolization.
A. Inhibition of late endosome fusion *in vitro* by anti-syntaxin 7 antibodies. For details, see *Experimental procedures*. Values, expressed as a percentage of the control (vesicles not incubated with anti-syntaxin 7 antibodies), are the mean of two experiments. Bars represent SE.
B and C. Effect of microinjected anti-syntaxin 7 antibodies on VacA-induced vacuolization. HeLa cells, microinjected with the antibodies, were then incubated for 3 h with 100 nM VacA before immunofluorescence staining with a secondary Texas red-conjugated antibody.

compartments from which they originate. Although part of these internal membranes may have been disrupted during sample preparation, their complete disappearance is evident. A retrospective analysis of previous electron microscopic observations of VacA-induced vacuoles (Catrenich and Chestnut, 1992; Cover *et al*., 1992; Ricci *et al*., 1997) further supports this observation.

On the basis of the present findings, we would like to propose that a major proportion of the limiting membranes of vacuoles derive from inner membranes that have fused

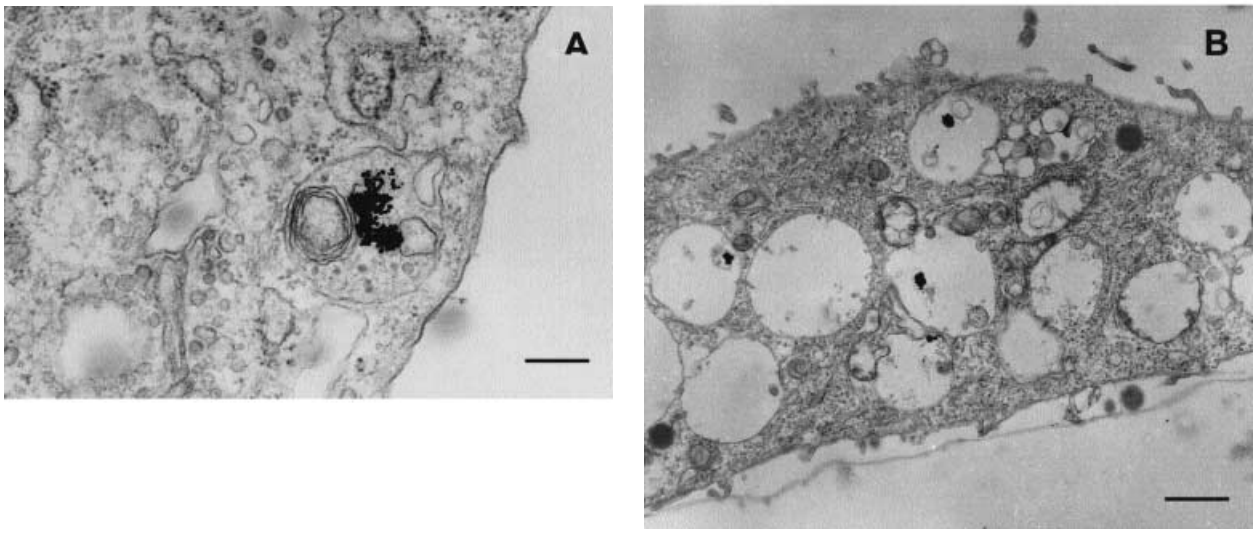

Fig. 4. Electron micrographs of vacuolated HeLa cells. Cells were fed with 15 nm gold–BSA for 4 h, chased overnight and then incubated with 100 nM VacA for 1 h (A) or 3 h (B) before processing for electron microscopy. BSA–gold previously accumulated in late endosomes and lysosomes appears inside vacuoles as large black clumps. Apart from the gold, note the sparseness of internal membranes in the vacuolar lumen (B). Bar (A): 1 µm. Bar (B): 200 nm.

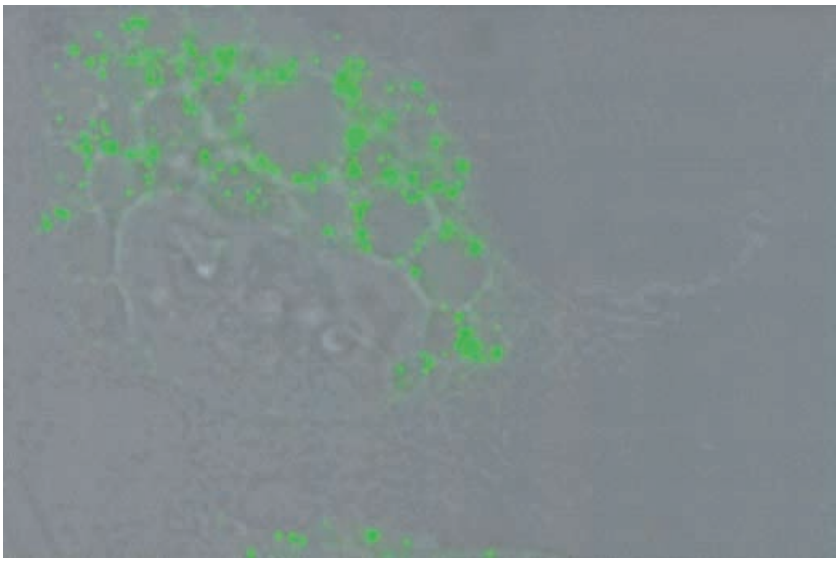

Fig. 5. LBPA distribution in vacuolated cells. HeLa cells were incubated for 4 h with 100 nM VacA in DMEM, 2% FCS plus 5 mM NH_4Cl before processing for immunofluorescence. Cells were stained with anti-LBPA monoclonal antibody. Transmission light and fluorescence images are superimposed. Magnification: 1000×.

with the cytosolic membrane of late endosomes and lysosomes in a rab7-dependent and SNARE-independent process. A valuable monoclonal antibody specific for the lyso-bisphosphatidic acid (LBPA) component of the internal endosomal and lysosomal membranes has been described (Kobayashi *et al*., 1999). As shown in Fig. 5, taken on HeLa cells at an intermediate stage of vacuolization, this antibody does not stain the lumen of swollen vacuoles, whereas it does stain small endosomal structures, which appear as intensively stained round structures adhering to the vacuole-limiting membrane. This pattern of staining does not exclude the possibility that limited amounts of LBPA are present on the external vacuole membrane. However, it appears that almost all the original content of LBPA has largely disappeared during vacuolization. This is not unexpected, as one role of the cone-shaped lipids such as LBPA is that of preventing membrane fusion (Chernomordik *et al*., 1995), and a major function of LBPA could therefore be that of preventing the fusion of the closely packed internal membranes of endosomes and lysosomes. If this is indeed the case, LBPA has to be metabolized in order for the fusion of internal endosomal membranes to occur. This process may take place in two different ways depending on the existence of discontinuity or continuity between the internal membranes and the limiting membrane of late endosomal compartments. In the former case, the internal membranes will have to fuse with the limiting membrane to form a continuous single membrane. Alternatively, if it is assumed that internal membranes are convoluted and folded invaginations of the external membrane (Griffiths, 1996), vacuolation might result from the unfolding of the invaginations of the peripheral endosomal membrane driven by the increasing osmotic pressure of the lumen, caused by the combined action of the anion-selective VacA channel and the electrogenic vacuolar ATPase proton pump (Montecucco and Rappuoli, 2001). In this context, the inhibitory effect on vacuolization observed in cells transfected with dominant-negative mutants of Rab7 (Papini *et al*., 1997), could be explained by considering that these mutants induce late endosome fragmentation (Meresse *et al*., 1995): as result, these smaller compartments are not capable of swelling into large vacuoles.

Experimental procedures

Reagents

Plasmid pGEMMyc-α-SNAP(L294A) was a kind gift from H. Stenmark (Norwegian Radium Hospital, Oslo, Norway); plasmid pGEMp95 has been described elsewhere (de Bernard *et al*., 1997). Polyclonal antibody against syntaxin 7 was kindly provided by J. P. Luzio (Cambridge Institute for Medical Research, Cambridge, UK). Monoclonal antibody 6C4 anti-LBPA was kindly provided by J. Gruenberg (University of Geneva, Switzerland). An affinity-purified polyclonal antibody against native VacA was kindly provided by J. Telford (Chiron, Siena, Italy). Monoclonal antibody against Myc tag was from Roche; peroxidase, Texas red- and fluorescein isothiocyanate (FITC)-conjugated antibodies to rabbit and mouse IgG were from Sigma. VacA was purified from an acellular culture filtrate of *H. pylori*, strain 17874 (Manetti *et al*., 1995), filter sterilized and stored at 4°C in 50 mM NaP_i, pH 7.4, 145 mM NaCl (phosphate-buffered saline, PBS). VacA was activated by acid pH treatment immediately before use as described previously (de Bernard *et al*., 1995), and all vacuolization assays were carried out in DMEM, 2% FCS, 5 mM NH_4Cl.

Transfection

HeLa cells seeded on coverslips the day before the experiment in a 24-well plate were washed once with internalization medium (IM: DMEM, 20 mM HEPES, pH 7.4), incubated with IM

containing recombinant vaccinia virus vT7 (0.1%) expressing the T7 polymerase for 30 min at room temperature and transfected in DMEM containing 3.7 g l^{-1} $NaHCO_3$, 10 mM HEPES, pH 7.2, 10 mM hydroxyurea, DNA (9 ng μl^{-1}) and DOTAP (27 ng μl^{-1}) for 2 h at 37°C (Stenmark *et al.*, 1995). Cells were then washed three times in DMEM plus 3.7 g l^{-1} $NaHCO_3$, 10% FCS and 10 mM hydroxyurea, pH 7.2, and incubated further in DMEM, pH 7.2, plus 2% FCS, 10 mM hydroxyurea and 5 mM NH_4Cl. Co-transfections were performed using 9 ng μl^{-1} each DNA mixed with 30 ng μl^{-1} DOTAP. Transfection efficiency was determined after indirect immunofluorescence staining with a monoclonal antibody anti-myc tag by calculating the percentage of positive cells in several randomly chosen fields.

Peroxidase internalization

On the day before the experiment, HeLa cells were seeded on 24-well plates and then transfected with pGEMMyc-α-SNAP(L294A). After 12 h of expression, cells were washed and incubated with 2 mg ml^{-1} peroxidase in DMEM, 2% FCS at 37°C for a further 2 h. After washing with PBS, cells were dissolved in 200 µl of PBS, 0.5% Triton X-100. Peroxidase activity was measured in 50 mM sodium phosphate buffer, pH 5.0, 0.1% Triton X-100, 0.1 mg ml^{-1} *o*-dianisidine, 0.003% H_2O_2. An aliquot of 500 µl of this solution was added to 5 µl of cell extract (clarified by centrifugation). After 5 min, the absorbance at 405 nm was measured. Control experiments were performed with cells transfected under the same conditions with pGEMMyc. All experiments were run in duplicate.

Immunofluorescence microscopy

Cells, seeded on coverslips, were fixed for 10 min in 4% paraformaldehyde in PBS and permeabilized in 100% methanol at –20°C for 20 s. Cells were then incubated with primary antibodies, such as anti-Myc, anti-VacA and anti-LBPA, diluted in PBS, 3% BSA for 1 h. After washing, they were incubated with secondary antibodies linked to fluorescein or Texas red. After a 30 min incubation, coverslips were mounted on glass slides. Cells were observed with a confocal laser scanning microscope (model MRC 1024; Bio-Rad Laboratories).

Cell-free assay of late endosome fusion

HeLa cells were incubated with either biotinylated horseradish peroxidase (HRP; 2 mg ml^{-1}) or avidin (4 mg ml^{-1}) in IM (DMEM, 20 mM Na HEPES, pH 7.5) for 10 min followed by a 40 min chase in IM at 37°C. Cells were scraped in HB (250 mM sucrose, 3 mM imidazole, pH 7.4), homogenized in the same medium by passage through a 22 gauge syringe needle and centrifuged at 1200 g for 10 min. The supernatants (PNS) were mixed separately with 60 µg ml^{-1} (final concentration) anti-syntaxin 7 antibody for 15 min at 4°C before fusion; they were mixed in the presence of 3 mg ml^{-1} cytosolic proteins (prepared from control cells after ultracentrifugation of PNS) and an ATP-regenerating system (2 mM ATP, 16 mM creatine phosphate and 80 µg ml^{-1} creatine phosphokinase) and 50 mg ml^{-1} biotinylated insulin in 12.5 mM Na HEPES, pH 7.5, 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, 50 mM KOAc. After 30 min, membranes were solubilized with 0.2% Triton X-100, and HRP activity was quantified (Beaufay *et al.*, 1974) after immunoprecipitation of avidin–biotin–HRP complexes (Gruenberg *et al.*, 1989).

Determination of NRU

In samples run in triplicate, cells were incubated in 5 mM neutral red diluted in PBS, 0.3% BSA for 8 min. After three washes in PBS, 0.3% BSA, neutral red was extracted with 70% ethanol and 0.37% HCl, and the absorbance at 540 nm was determined.

Microinjection

Cells were microinjected using Eppendorf microcapillaries. The micropipette was controlled using an Eppendorf micromanipulator attached to a Zeiss inverted microscope. Injection pressure was controlled using an Eppendorf microinjector model 5240. Polyclonal antibody against syntaxin 7 was clarified before injection by centrifugation for 15 min at 16 000 *g*. Cells were then incubated for 3 h with 100 nM VacA before being processed for immunofluorescence.

Electron microscopy

HeLa cells were incubated with 16 nm gold–BSA for 4 h, washed and chased overnight in DMEM–HEPES and then exposed to 100 nM VacA for 1 or 3 h. Cells were fixed with 1% glutaraldehyde in 0.2 M HEPES, pH 7.4, for 1 h and post-fixed in 1% osmium tetroxide for 15 min, followed by dehydration in a graded ethanol series. At the 70% stage, the preparations were left in a saturated solution of uranyl acetate in 70% ethanol. The cells were embedded *in situ* in epoxy resin and sectioned. Cells were observed with a Philips 400T electron microscope.

Acknowledgements

We thank E. Papini and A. Piccin for many discussions and for critical reading of the manuscript. We thank J. P. Luzio for anti-syntaxin 7 antibody, J. Gruenberg for anti-LBPA antibody, H. Stenmark for the gift of pGEMMyc-α-SNAP(L294A) and J. Telford for anti-VacA antibody. This work was supported by MURST 40% Project on Meccanismi e segnali nel trasporto cellulare di proteine, CNR-MURST Biotecnologie legge 95/95 and the Armenise-Harvard Medical School Foundation.

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