

The rab7 GTPase controls the maturation of *Salmonella typhimurium*-containing vacuoles in HeLa cells

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Following entry into non-phagocytic HeLa cells, the facultative pathogen *Salmonella typhimurium* survives and replicates within a membrane-bound vacuole. Preceding the initiation of intracellular replication there is a lag phase, during which the bacteria modulate their environment. This phase is characterized by the rapid recycling of early endosomal proteins present on the nascent vacuole followed by the acquisition of a subset of lysosomal proteins. To gain a better understanding of the mechanism of intracellular survival, we have followed the biogenesis of the *S.typhimurium*-containing vacuole (SCV) in HeLa cells expressing different mutant forms of the small GTPase rab7. We demonstrate that the SCV recruits pre-existing lysosomal glycoproteins (Lgps) in a rab7-dependent manner, without directly interacting with lysosomes. We also show the transient accumulation, in the vicinity of the SCV, of novel rab7- and Lgp-containing vesicles containing very low amounts of cathepsin D. The size of these vesicles is dependent on rab7 activity, suggesting a role for rab7 in their homotypic fusion. Taken together, these results indicate that rab7 regulates SCV biogenesis during the phase characterized by the rapid acquisition of lysosomal proteins. We propose that SCV maturation involves its interaction with rab7/Lgp-containing vesicles which are possible intermediate cargo components of the late endocytic pathway.

Keywords: flow cytometry/lysosome/rab7/*Salmonella typhimurium*/vacuole

Introduction

Upon entering a host cell, many intracellular pathogens reside within membrane-bound vacuoles. However, most bacteria phagocytosed by macrophages are killed by the rapid delivery into the phagosome of hydrolytic enzymes and other bactericidal molecules. Successful intracellular parasitism is dependent on the pathogen-driven control of the biogenesis of the vacuole allowing the establishment of a proper niche for survival and replication. Certain bacteria such as *Shigella* or *Listeria monocytogenes* rapidly lyse the vacuole membrane and escape into the nutrient-

rich cytoplasm. Others have developed sophisticated strategies to control the maturation of their vacuole (Finlay and Cossart, 1997). For example, *Legionella pneumophila*- and virulent *Brucella abortus*-containing vacuoles fuse with nascent autophagosomes and replicate in the endoplasmic reticulum (ER) (Swanson and Isberg, 1995; Pizarro-Cerda *et al.*, 1998a,b). *Mycobacterium tuberculosis* and *Leishmania donovani* promastigotes remain in an early endosomal vacuole by inhibiting the process of phagolysosome maturation (Desjardins and Descoteaux, 1997; Via *et al.*, 1997). The molecular basis of these mechanisms that pathogens have developed to control the vacuole biogenesis remains essentially unknown.

Salmonella typhimurium is a facultative intracellular pathogen that triggers its entry into both phagocytic and non-phagocytic cells by macropinocytosis (Francis *et al.*, 1993). It remains in a vacuole within which it replicates after a lag period of several hours. In a recent study, we analysed very early events in the biogenesis of *Salmonella*-containing vacuoles (SCVs) in epithelial cells (Steele-Mortimer *et al.*, 1999). We showed that the SCV acquires certain markers of the early endosome, the recycling compartment and the lysosome. This biogenesis is characterized by a rapid loss of the early endocytic markers EEA1 and transferrin receptor (TfR) and by a progressive enrichment in the vacuolar ATPase and some lysosomal glycoproteins (Lgps). The SCV acquires lysosomal molecules such as lamp1, lamp2 and lysosomal-acid phosphatase, but is essentially devoid of lysosomal markers whose targeting relies on the presence of the mannose 6-phosphate signal (most lysosomal enzymes) and also of the mannose 6-phosphate receptors (MPRs) themselves (Garcia-del Portillo and Finlay, 1995). The rapid acquisition of early endosomal markers suggests a direct fusion of the nascent vacuole with early endosomes. However, there does not appear to be a direct interaction between SCVs and late endosomal compartments. In addition to excluding soluble lysosomal enzymes, the SCV shows a limited accessibility to fluid phase endocytic probes and mature lysosomes in which a fluid phase marker had been pre-loaded (Garcia-del Portillo and Finlay, 1995). Thus, interactions of the SCV with intracellular compartments and the mechanism by which it captures and becomes enriched in a specific subset of lysosomal proteins remain poorly understood.

There is now substantial evidence that GTPases of the rab family are crucial regulators of phagosome and vacuole biogenesis. These small GTP-binding proteins are recruited specifically to the cytoplasmic face of distinct organelles in both endocytic and secretory pathways and control both spatial and temporal partnering of vesicles with their target membranes (Rybin *et al.*, 1996; Novick and Zerial, 1997). rab5, which regulates homotypic fusion of early endosomes (Gorvel *et al.*, 1991), is present on the membrane of latex

bead-containing phagosomes in macrophages (Rabinowitz *et al.*, 1992; Desjardins *et al.*, 1994) and on the vacuoles formed in *Mycobacterium bovis*-infected cells (Via *et al.*, 1997). It also regulates the fusion competence of *L. monocytogenes*-containing vacuoles with endosomes *in vitro* (Alvarez-Dominguez *et al.*, 1996). Concomitant with the loss of rab5 during the maturation of latex bead-containing phagosomes into phagolysosomes, the late endosome-associated rab7 GTPase is incorporated progressively (Desjardins *et al.*, 1994). Indeed, the failure of mycobacterial vacuoles to undergo fusion with lysosomes has been attributed to the selective exclusion of rab7 from these vacuoles (Via *et al.*, 1997).

rab7 functions in the endocytic pathway downstream of rab5 (Mukhopadhyay *et al.*, 1997) and is necessary for the delivery of endocytosed material to lysosomes (Vitelli *et al.*, 1997). However, it remains unclear at exactly which transport steps it is involved. We have shown previously that a GTPase-defective mutant of rab7 is partially redistributed from a pre-lysosomal compartment to lysosomes, suggesting that it plays a role in vesicular trafficking to lysosomes (Mésesse *et al.*, 1995). In contrast, delayed processing of SV5 hemagglutinin-neuraminidase and of procathepsin D, accompanied by the redistribution of the cation-independent MPR (CI-MPR) from perinuclear to large peripheral endosomes, in cells expressing dominant-negative forms of rab7 led Wandinger-Ness and colleagues (Feng *et al.*, 1995; Press *et al.*, 1998) to favour a more upstream effect: regulating early to late endosome transport. Finally, rab7 has been found to modulate the homotypic fusion between late endosomes in a cell-free system (Papini *et al.*, 1997). These data suggest that rab7 plays a central role in transport steps connecting late endosomes to upstream and downstream endocytic compartments and possibly to the phagocytic route.

Considering the indications for rab protein involvement in vacuole development, we designed a system to investigate the role of rab7 in SCV biogenesis. HeLa cells expressing various mutant forms of rab7 were infected with *S. typhimurium*. The presence on the SCV of key markers of the maturation process was assessed by immunofluorescence confocal microscopy and quantified by a recently developed epitope-specific flow cytometric method. We show that rab7 is present on SCVs during the biogenesis phase, which is characterized by the enrichment in some Lgps and that the kinetics of maturation of the SCV is retarded in cells overexpressing a dominant-negative form of the rab7 GTPase. On the basis of these results, we postulate that SCVs mature and are enriched in pre-existing lysosomal glycoproteins by a rab7-mediated vesicular transport step.

Results

Inducible expression of rab7 mutants in stable HeLa cells transfectants

In order to be able to regulate expression and thus circumvent the toxicity problems often encountered with stable expression systems, we prepared HeLa cell lines which expressed rab7 proteins under a tetracycline-regulated inducible system (Gossen and Bujard, 1992; Méresse *et al.*, 1995). As well as wild-type rab7, we used dominant-negative (T22N) and GTPase-defective (Q67L) mutant

forms of rab7, which are analogous to the inhibitory and activating forms of p21-ras, respectively. Cells, referred to as rab7-WT-, rab7-Q67L- or rab7-T22N-HeLa cells, were grown in the presence of anhydrotetracycline (ATc). ATc binds to the transactivator, thereby inhibiting its binding to the DNA and subsequent transcription and translation. Growing cells without ATc for 72 h caused a 3- to 6-fold expression of recombinant proteins over endogenous rab7 (Mésesse *et al.*, 1995).

Expression of rab7-T22N delays the maturation of SCV

We recently optimized the efficiency of invasion of HeLa cells by *S. typhimurium* (Steele-Mortimer *et al.*, 1999). Using this protocol (see Material and methods), virtually every cell could be infected with 1–10 bacteria using short (5–10 min) invasion times. The presence on SCVs of EEA1 and lamp1, used as markers for early endosomes and lysosomes, respectively, was analysed by immunofluorescence in HeLa and rab7-T22N-HeLa cells. Cells were infected for 5 min with green fluorescent protein (GFP)-expressing *S. typhimurium* (GFP-*S. typhimurium*) and either processed immediately for immunofluorescence or incubated further for up to 60 min in growth medium in the presence of gentamicin. As previously described (Steele-Mortimer *et al.*, 1999), EEA1 but not lamp1 was present on SCVs after 5 min invasion of HeLa cells (Figure 1A–C). The same rapid and selective recruitment of EEA1 was observed in cells expressing the dominant-negative mutant of rab7 (Figure 1D–F). In control HeLa cells, EEA1 was then rapidly removed from the SCV whereas lamp1 was recruited (Figure 1G–I). In contrast, we observed that in rab7-T22N-HeLa cells, most SCVs were negative for both EEA1 and lamp1 at 1 h post-infection (p.i.) (Figure 1J–L). This result suggests that the expression of a dominant-negative form of the rab7 GTPase alters the maturation process of SCVs.

In order to confirm this observation and to define and quantify this phenomenon more accurately, we used an epitope-specific flow cytometric method to analyse the presence of specific markers in a large population of individual SCVs (Mésesse *et al.*, 1997). Using this approach, we previously have shown that TfR, a marker of the recycling compartment (Mayor *et al.*, 1993), is rapidly recruited by SCVs although with slightly different kinetics than EEA1 (Steele-Mortimer *et al.*, 1999). The percentages of SCVs containing either TfR but not lamp1 (TfR⁺/lamp1⁻), lamp1 but not TfR (TfR⁻/lamp1⁺) or containing both markers (TfR⁺/lamp1⁺) were then determined. Figure 2A shows that, at 10 min p.i. in HeLa cells, 50% of SCVs were TfR⁺/lamp1⁻ and 5% were TfR⁻/lamp1⁺. Conversely, at 60 min p.i., only 4% of SCVs were TfR⁺/lamp1⁻ and 71% were TfR⁻/lamp1⁺. The percentage of double-positive (TfR⁺/lamp1⁺) SCVs changed from 45 to 25% during this period of time (Figure 2B) and the relative amounts of TfR and lamp1 in this population of SCVs varied dramatically (Figure 2C). At 10 min p.i., TfR⁺/lamp1⁺ SCVs were highly enriched in TfR, whereas lamp1 was the predominant marker at later time points. This analysis was also performed on vacuoles prepared from cells expressing the dominant-negative form of rab7. When compared with control cells, less TfR⁻/lamp1⁺ and more TfR⁺/lamp1⁻ SCVs were found, especially at 40 and 60 min p.i. In addition, although

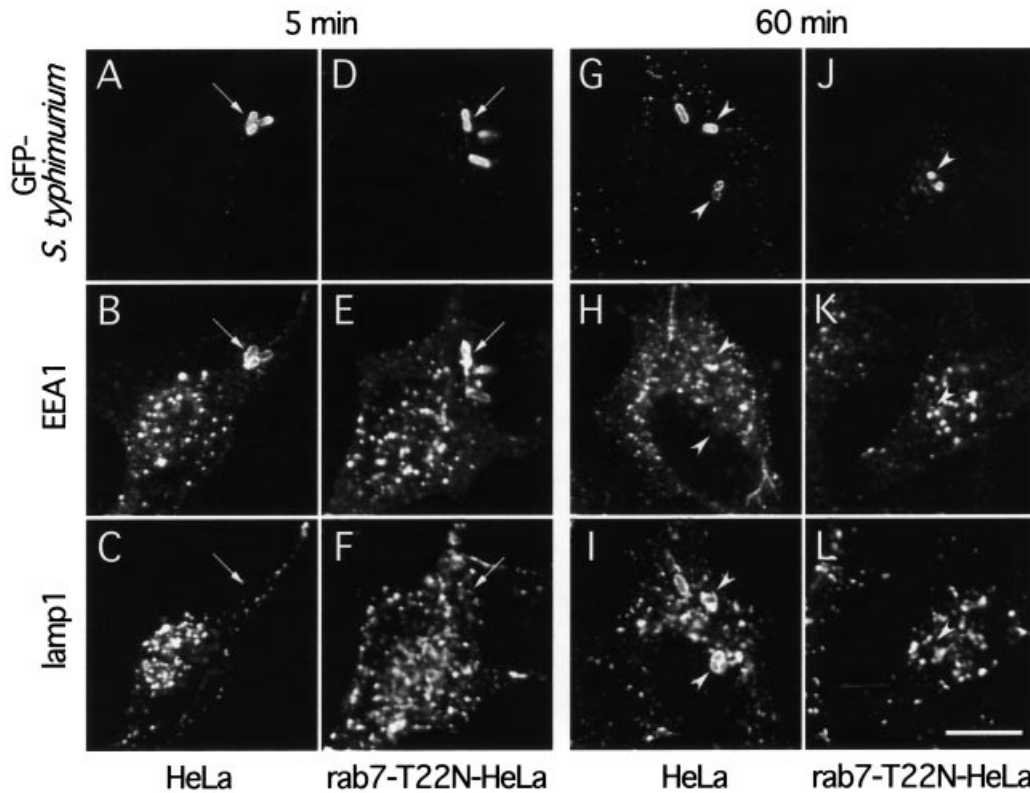


Fig. 1. SCV membranes rapidly acquire EEA1 but fail to accumulate lamp1 in rab7-T22N-HeLa cells. HeLa (A–C and G–I) or rab7-T22N-HeLa (D–F and J–L) cells were infected with GFP-*S.typhimurium* for 5 min and then either fixed immediately (A–F) or washed and incubated further to 60 min p.i. (G–L). Cells were processed for double immunolabelling and three-colour confocal microscopy as described in Materials and methods. (A, D, G and J) GFP-*S.typhimurium*. (B, E, H and K) Distribution of EEA1. (C, F, I and L) Distribution of lamp1. At 5 min p.i., EEA1 but not lamp1 were present on SCVs in HeLa (arrow in A–C) and rab7-T22N-HeLa cells (arrow in D–F). At 60 min p.i., lamp1 but not EEA1 were present on SCVs formed in HeLa cells (arrowheads in G–I) whereas neither lamp1 nor EEA1 were seen on SCV membranes in rab7-T22N-HeLa cells (arrowhead in J–L). Bar, 10 μ m.

numbers of Tfr⁺/lamp1⁺ SCVs were comparable in both cell lines (Figure 2B), the maturation of this population of vacuoles in rab7-T22N-HeLa cells was strongly retarded. Both the recycling of Tfr and the acquisition of lamp1 were strongly inhibited (Figure 2C, compare mean fluorescences for Tfr and lamp1 at 20, 40 and 60 min in HeLa and rab7-T22N-HeLa). The relative levels of these markers were also compared in the total population of SCVs and the mean fluorescence of SCVs was compared between rab7-T22N- and control HeLa cells. As shown in Figure 2D, Tfr appeared to be removed less efficiently and lamp1 acquired more slowly in rab7-T22N-HeLa cells than in control cells, with maximum differences seen at 40–60 min p.i. For example, the mean fluorescence seen for lamp1 at 60 min p.i. in rab7-T22N-HeLa cells was about half of that in control HeLa cells. This value corresponds to the mean fluorescence measured in control HeLa cells at 20–30 min p.i. (Figure 2D), a period of time at which 15–30% of SCVs were scored as lamp1 positive by immunofluorescence (Steele-Mortimer *et al.*, 1999). This observation is also true for the Tfr⁺/lamp1⁺ SCV subpopulation (Figure 2C). At 2 h p.i., both Tfr and lamp1 contents tended to reach control values. Eventual completion of the maturation of SCVs is supported by the fact that bacterial replication was comparable in HeLa and rab7-T22N-HeLa cells (not shown). In summary, the flow cytometric analysis demonstrates that lamp1 acquisition by SCVs is delayed in cells expressing a dominant-negative form of rab7, and confirm the immuno-

fluorescence microscopy observations (Figure 1J–L). In addition, this technique also revealed that the dominant-negative mutant of rab7 causes an apparent decrease in the rate of recycling of Tfr from the SCV.

rab7 is transiently present on SCVs

We reasoned that if rab7 is involved in the maturation of the vacuole, it should be associated at some point with the SCV membrane. In order to test this hypothesis, post-nuclear supernatant (PNS) prepared from HeLa cells infected for different times with *S.typhimurium* was incubated with an antibody against rab7 and analysed by flow cytometry. Figure 3 shows that ~60% of SCV were rab7 positive at 40 min p.i. and that this percentage then decreases to 35% at 1 h. Considering that membrane-associated rab GTPases are recycled constantly to the cytoplasm in a GDI-bound form (Novick and Zerial, 1997), the maximum level of 60% rab7-positive SCV detected at 40 min p.i. is likely to reflect transient association of the GTPase with the vacuole membrane rather than a phenomenon that concerns only a fraction of the SCV population.

The presence of rab7 and Lggs on SCVs was also investigated by immunofluorescence. In HeLa cells infected for 40 min, most SCVs were rab7 positive and few of them were lamp2 positive (Figure 4D–F). We also observed clusters of rab7-containing vesicles in close proximity to vacuoles. This transient phenomenon was first detectable at 20 min p.i. (Figure 4A–C) but rarely observed at 60 min p.i. when,

by contrast, a strong labelling for lamp2 was seen (Figure 4G–I). Although rab7 could still be detected on 35% of SCVs by flow cytometry at 60 min p.i. (Figure 3), the level of rab7 was too low to be detected by immunofluorescence (Figure 4G–I). The presence of rab7 on SCVs and clustered rab7-containing vesicles were also observed in nocodazole-treated cells, suggesting that this process is independent of the microtubule network (not shown).

Immunolabellings were also carried out in rab7-WT and rab7-Q67L-HeLa cells infected for 30 min with *S.typhimurium*. In these cells, SCVs were heavily decorated with anti-rab7 antibodies and large clusters of rab7-containing vesicles were observed transiently between 20 and 40 min p.i. (Figure 5). Strikingly, overexpression of wild-type rab7 or the GTPase mutant increased the size of rab7-containing vesicles clustered in the vicinity of phagosomes

(Figure 5A–C and D–F). In contrast, neither the presence of rab7 on SCVs nor the clustering of rab7-containing vesicles was observed when rab7-T22N-HeLa cells were used (not shown). Together, these microscopic observations are in good agreement with the flow cytometric analysis of SCVs. They confirm that the rab7 content of SCVs is maximal at 30–40 min p.i. and that it precedes the acquisition of Lgps. In addition, these experiments revealed the presence of novel clusters of rab7-containing vesicles whose size appears to be controlled by rab7.

Clustered rab7-containing vesicles are highly enriched in lamp2 but not in cathepsin D

Our results suggested that rab7 is involved in the biogenesis of SCVs and we considered that surrounding SCVs with rab7-containing vesicles could favour a rapid enrichment

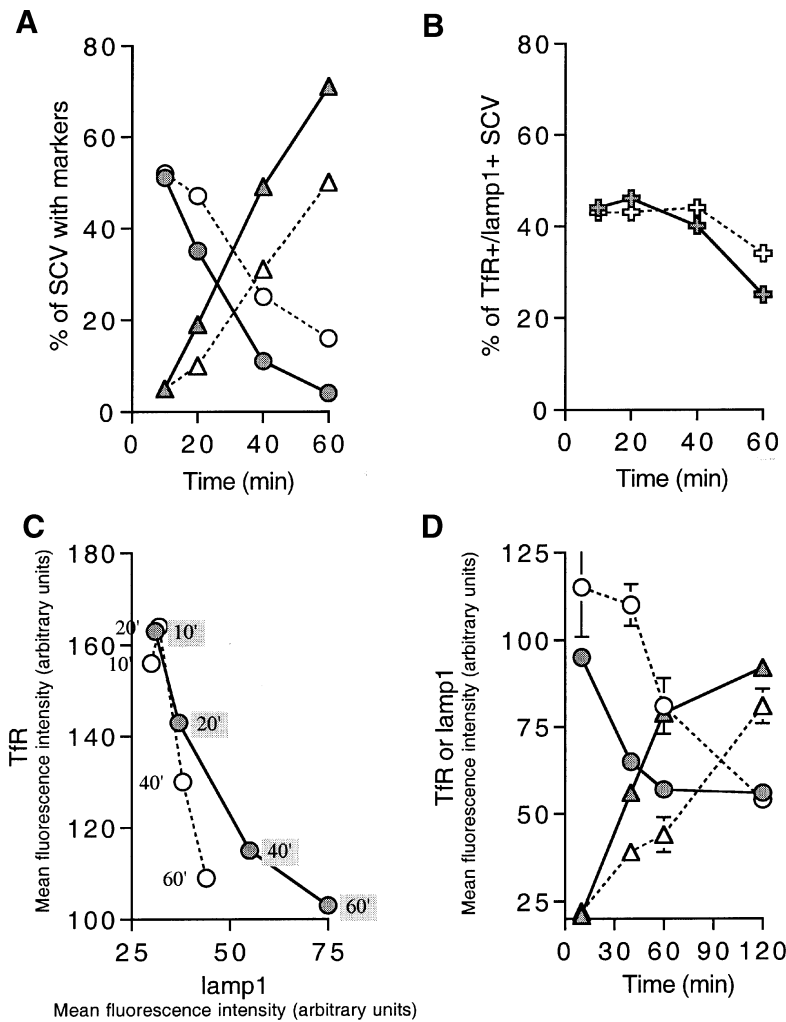


Fig. 2. Maturation of SCVs is delayed in rab7-T22N-HeLa cells. HeLa or rab7-T22N-HeLa cells were infected with GFP-*S.typhimurium* for 10 min, washed and either processed immediately or incubated further as indicated. PNS was prepared at different times p.i., immunolabelled with antibodies raised against the cytoplasmic domain of TIR and lamp1 followed by PE-conjugated donkey anti-mouse and Cy5-conjugated anti-rabbit secondary antibodies, and analysed by triple-colour flow cytometry. (A) The percentage of SCVs containing either TIR alone (circles) or lamp1 alone (triangles) was determined in HeLa (closed symbols) and rab7-T22N-HeLa cells (open symbols) and plotted as a function of time. (B) During the first hour of infection, the population of TIR⁺/lamp1⁺ SCVs decreased from 45% of the total to 25–35% in HeLa (closed crosses) and rab7-T22N-HeLa cells (open crosses). (C) TIR⁺/lamp1⁺ SCVs were analysed. The mean fluorescence intensity of TIR versus lamp1, which reflects the relative abundance of both epitopes, was plotted for the different time points in HeLa (●) or rab7-T22N-HeLa cells (○). Data from a typical experiment are presented. (D) The mean fluorescence of total SCVs for TIR (circles) or lamp1 (triangles) was compared in rab7-T22N-HeLa (open symbols) versus control HeLa cells (closed symbols) and plotted as a function of time. In order to compare several separate experiments, the mean fluorescence was first expressed as a percentage of the value in control HeLa cells and then normalized to a typical experiment performed with control HeLa cells. Values for rab7-T22N-HeLa cells are the mean of four experiments ± SD.

of phagosomes with late endocytic/lysosomal molecules. To test this hypothesis, HeLa cells infected for 30 min with *S.typhimurium* were fixed and immunolabelled for rab7, lamp2 and cathepsin D. rab7 and lamp2 co-localized on the clustered vesicles surrounding SCVs (Figure 6A–C; see also Figure 4) although they are not significantly co-localized elsewhere in HeLa cells (Méresse *et al.*, 1995). Cathepsin D is a luminal lysosomal enzyme which is essentially excluded from SCVs (Garcia-del Portillo and Finlay, 1995). Despite its extensive co-localization with

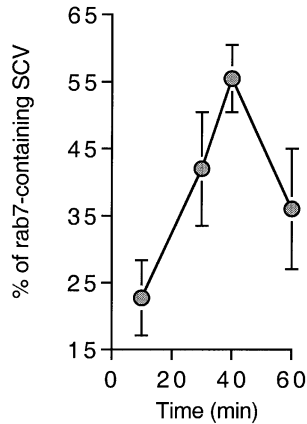


Fig. 3. rab7 transiently accumulates on SCVs. HeLa cells were infected with GFP-*S.typhimurium* for 10 min, washed and either processed immediately or incubated further for up to 60 min. PNS were prepared, immunolabelled with antibodies raised against rab7 and analysed by flow cytometry as previously described. Values are the means of three experiments \pm SD.

Lgps in lysosomes, very little cathepsin D could be detected in the clustered rab7⁺/lamp2⁺ vesicles (Figure 6D–F). The CI-MPR was similarly absent from vesicular clusters (not shown). These results suggest that infection of HeLa cells with *S.typhimurium* induces the clustering in the vicinity of SCVs of a novel compartment highly enriched in rab7 and Lgps but not in cathepsin D nor in CI-MPR.

Newly synthesized proteins are not essential for the biogenesis of SCVs

It has been proposed that Lgps are delivered to SCVs from the biosynthetic pathway (Garcia-del Portillo and Finlay, 1995). In order to test this hypothesis, protein synthesis was inhibited and newly synthesized proteins then chased from the ER/Golgi secretory apparatus. For this, HeLa cells were treated for 3 h with cycloheximide and then infected in its presence. PNS was prepared at different time points and the TfR and lamp1 content of SCVs was compared with that of cycloheximide-untreated cells. As shown in Figure 7, no difference was seen in the acquisition/recycling profiles of TfR and lamp1 in treated versus untreated cells. This result suggests that both TfR and lamp1 are acquired from the pool of pre-existing molecules and that the biosynthetic pathway does not play a major role in the biogenesis of SCVs.

Discussion

It has been shown that *S.typhimurium* survives and replicates within a novel vacuole that is highly enriched in a

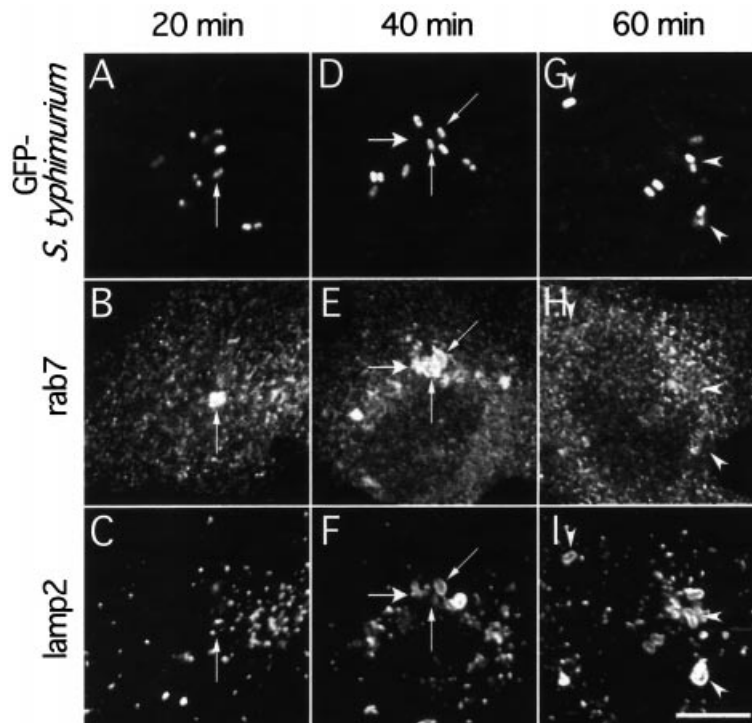


Fig. 4. rab7 is present on and at the periphery of SCVs. HeLa cells were infected with GFP-*S.typhimurium* as previously described, further incubated and fixed at either 20, 40 or 60 min p.i. Samples were processed for double immunolabelling and analysed by three-colour confocal microscopy. (A, D and G) GFP-*S.typhimurium*. (B, E and H) Distribution of rab7. (C, F and I) Distribution of lamp2. The presence of rab7 on SCVs was detected at 20 min p.i (arrow in B) and reached a maximum at 40 min p.i. (arrows in E). Clusters of rab7- and lamp2-containing vesicles are also seen at this time point (large arrow in D–F). Most SCVs were heavily labelled with the anti-lamp2 at 60 min p.i. whereas rab7 was no longer detectable (arrowheads in G–I). Bar, 10 μ m.

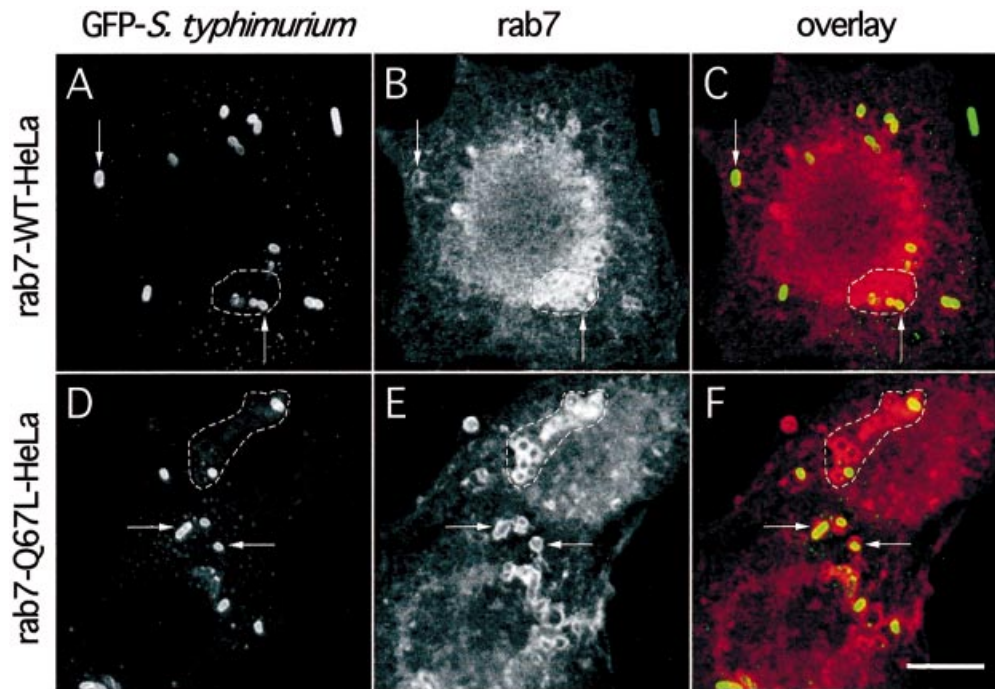


Fig. 5. Clustered rab7-containing vesicles are larger in rab7-WT- and rab7-Q67L-HeLa cells. HeLa cells overexpressing rab7-WT (A–C) or rab7-Q67L (D–F) were infected with GFP-*S.typhimurium* as previously described and fixed at 30 min p.i. Samples were processed for immunolabelling and analysed by two-colour confocal microscopy. (A and D) GFP-*S.typhimurium*. (B and E) Distribution of rab7. (C and F) Colour images obtained by merging GFP (green) and rab7 (red) images. SCVs are decorated by anti rab7 antibodies in rab7-WT-HeLa (arrows in A–C) and in rab7-Q67L-HeLa cells (arrows in D–F). Clustered rab7-containing vesicles appear enlarged both in rab7-WT-HeLa and rab7-Q67L-HeLa cells (area delimited by the dotted line in A–C and D–F, respectively). Bar, 10 μ m.

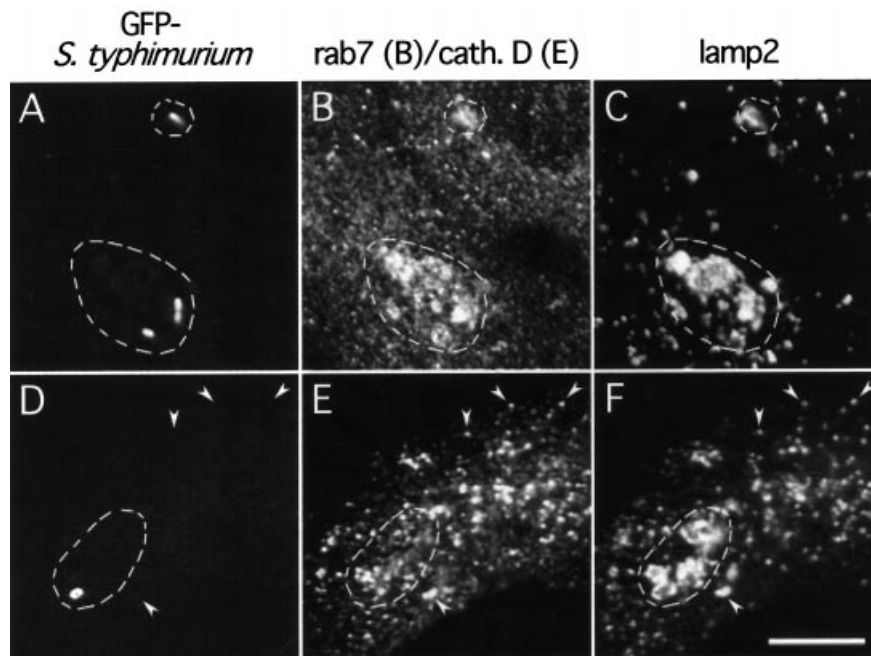


Fig. 6. Clustered rab7-containing vesicles are positive for lamp2 but essentially devoid of cathepsin D. HeLa cells were infected with GFP-*S.typhimurium* as previously described and fixed at 30 min p.i. Samples were processed for double immunolabelling and analysed by three-colour confocal microscopy. (A and D) GFP-*S.typhimurium*. (B) Distribution of rab7. (E) Distribution of cathepsin D. (C and F) Distribution of lamp2. SCVs (A) were surrounded by rab7- (B) and lamp2- (C) containing vesicles (area delimited by dotted lines, A–C). Although cathepsin D and lamp2 co-localize extensively in HeLa cells (arrowheads in E and F), cathepsin D is not concentrated together with lamp2 in clustered vesicles surrounding SCVs (area delimited by the dotted line, D–F). Bar, 20 μ m.

subset of Lgps and that intracellular replication is preceded by a lag phase during which the composition of the SCV membrane changes (Garcia-del Portillo *et al.*, 1993). Although a direct fusion of the nascent SCV with early

endocytic compartments may explain the very rapid acquisition of early endosomal proteins, the origin and mechanism of acquisition of Lgps present on SCVs still remain to be determined. Here, we show that SCVs capture

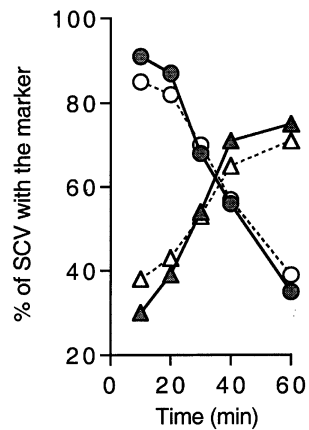


Fig. 7. Cycloheximide treatment of HeLa cells does not influence the biogenesis of SCVs. HeLa cells were treated (open symbols) or not (closed symbols) for 3 h with cycloheximide and infected with GFP-*S.typhimurium*. PNS were prepared at different time p.i. and SCVs were analysed for their content in Tfr (circles) and lamp1 (triangles). No significant differences were observed for the kinetics of acquisition/recycling of Tfr and lamp1 between cycloheximide-treated and untreated cells. Data from a typical experiment are presented.

Lgps from a pre-existing pool by a mechanism which requires the small GTP-binding protein rab7. We propose that infection by *S.typhimurium* triggers the transient accumulation of coalescent rab7- and lamp1-containing vesicles and that these vesicles mediate the efficient and rapid maturation of SCVs.

In contrast to phagosomes containing inert particles (Pizarro-Cerda *et al.*, 1998b), SCV acquires a subset of lysosomal molecules without interacting directly with either the MPR-containing late endosome or the lysosome. This novel pathway of vacuole biogenesis was first suggested by the finding that both MPRs and soluble lysosomal content are largely excluded from SCVs, and secondly by the demonstration that SCVs are not readily accessible to endocytosed probes (Garcia-del Portillo and Finlay, 1995). Based on these observations, it has been suggested that Lgp-rich vesicles arising from the secretory pathway could be involved in delivery of Lgp to the SCV (Garcia-del Portillo and Finlay, 1995). This implies that the SCV would essentially be enriched in newly synthesized Lgps and that inhibition of host cell protein synthesis would inhibit SCV maturation. However, in this study, we show that inhibition of host cell protein synthesis with cycloheximide does not affect the maturation profile of either Tfr or lamp1 on SCVs (Figure 7). Most *de novo* synthesized proteins complete their passage through the secretory system within 1 h. Human lamp1, which contains 18 *N*-linked carbohydrate chains, becomes resistant to endoH treatment with a $t_{1/2} = 45$ min, indicating completion of passage through the Golgi apparatus (Carlsson *et al.*, 1988). It is therefore unlikely that residual lamp1 present in the Golgi after >3 h of cycloheximide treatment could be responsible for the SCV maturation we observed in the presence of this drug. It is also known that, while the vast majority of Lgps are localized in lysosomes at steady state (Lippinott-Schwartz and Fambrough, 1987; Geuze *et al.*, 1988), they are recycled rapidly from the lysosome to the cell surface and endosomes (Lippinott-Schwartz and Fambrough, 1987; Wubbolts *et al.*, 1996). We conclude from these experiments that SCVs do not

acquire Lgps from the biosynthetic pathway, but rather from the endocytic pathway along which they recycle.

The rab5 and rab7 GTPases are key regulators of vesicular transport along the endocytic pathway (for reviews, see Novick and Zerial, 1997; Schimmoller *et al.*, 1998), and have been shown or suggested to be involved in the biogenesis of phagosomes and vacuoles. Phagosomes containing inert particles acquire rab5 and rab7 sequentially (Desjardins *et al.*, 1994). However, live intracellular pathogens appear to be able to regulate the association of these proteins with the vacuole membrane. For example, *M.tuberculosis*-containing vacuoles accumulate rab5 but fail to acquire rab7 (Via *et al.*, 1997), and *L.donovani* promastigotes prevent the fusion of their vacuole with compartments containing lysosomal enzymes. In the latter example, the arrest in maturation has been attributed to the selective exclusion of rab7 from the vacuole membrane (Scianimanico *et al.*, 1999). A direct role for rab5 in vacuole biogenesis was demonstrated for *L.monocytogenes*-containing vacuoles which require this protein for fusion with endosomes (Alvarez-Dominguez *et al.*, 1996). Here we show that the acquisition of rab7 by the SCV precedes that of lamp1 (Figure 4). The percentage of rab7-positive SCVs is highest at a period which is characterized by a rapid enrichment in Lgps (Figures 2 and 3), suggesting that rab7 may play a role in the biogenesis of the SCV. This was confirmed when expression of the dominant-negative form of rab7 was shown to delay acquisition of lamp1 (Figures 1 and 2). Several studies in mammalian cells have suggested that rab7 is required at different steps of the endocytic pathway (Méresse *et al.*, 1995; Press *et al.*, 1998). Our results show for the first time that rab7 also acts as a regulator of SCV biogenesis.

The rapid acquisition of EEA1 and Tfr in rab7-T22N-HeLa cells indicates that interactions between SCVs and the early endosomal compartment are not influenced by this GTPase. However, the defect in lamp1 acquisition observed in these cells was coupled to an increase in the persistence of the Tfr on the vacuole. This may reflect a defect in a hitherto undescribed rab7-controlled vesicle-mediated recycling pathway for early markers. Alternatively, maturation of the SCV could be mediated by multiple transient interactions with a competent late endocytic organelle as proposed in the 'kiss and run' model (Desjardins, 1998). Such transient interactions would favour bi-directional exchanges of membranous and luminal content. According to this model, the disruption of such a rab7-mediated fusion event would account for both delays in acquisition of lamp1 and loss of Tfr.

Beyond the discussion of models of vacuole biogenesis, a crucial question remains: what is the compartment whose interactions with the SCV is dependent on rab7 and that triggers SCV maturation? We have observed, in infected cells, a transient clustering of rab7⁺/Lgp⁺ vesicles that were not enriched in cathepsin D and CI-MPR. Interactions of SCVs with these clusters could account for the acquisition of subsets of late endosomal (rab7 but not CI-MPR) and lysosomal molecules (Lgps but not cathepsins). This phenomenon was not observed in rab7-T22N cells in which maturation of the vacuole is delayed but eventually occurs. This result indicates that in spite of the expression of the dominant-negative mutant, endogenous rab7 is capable of supporting a slow SCV maturation. It also

suggests that the transient accumulation of rab7⁺/Lgp⁺ vesicles in the close proximity of SCVs favours rapid SCV maturation. Interestingly, recent data suggest that Lgps and lysosomal enzymes are transported independently to lysosomes by AP1- and AP3-coated vesicles, respectively, giving a molecular basis for the dissociated targeting of these two classes of molecules (Le Borgne and Hoffack, 1998; Le Borgne *et al.*, 1998). Also, a consensus of data is consistent with the idea that intermediate vesicles connect CI-MPR⁺/rab7⁺ late endosomes to Lgp⁺/lysosomal hydrolase⁺ lysosomes. Co-localization of rab7 with the CI-MPR in late endosomes has been demonstrated (Chavrier *et al.*, 1990) and recently supported by the mislocalization of this receptor upon expression of a dominant-negative form of rab7 (Press *et al.*, 1998). However, both optical (Méresse *et al.*, 1995; Vitelli *et al.*, 1997) and electron microscopic (Rabinowitz *et al.*, 1992) observations have shown the existence of a rab7⁺/CI-MPR⁻ compartment. It has also been shown recently that the phosphatidylinositol 3-kinase inhibitor wortmannin causes the redistribution of both Lgp110 and Lgp120 to CI-MPR⁻/cathepsin L⁻ endosomal structures in NRK cells (Reaves *et al.*, 1996). The authors proposed that Lgps recycling from lysosomes to CI-MPR⁻ late endosomes accumulate in this compartment as the result of the inhibition of their traffic to lysosomes by wortmannin. Intoxication of mammalian cells with VacA, a toxin produced by *Helicobacter pylori*, induces the formation of a rab7⁺/Lgp⁺/CI-MPR⁻/lysosomal hydrolase⁻ compartment (Papini *et al.*, 1994; Molinari *et al.*, 1997). Considering the markers present, the VacA-induced compartment is very similar to the clustered rab7- and Lgp-containing vesicles we observed in *S.typhimurium*-infected cells. Together, these results strongly suggest the existence of vesicular trafficking pathways between late endocytic compartments whose inhibition may result in an abnormal segregation of their resident markers. We hypothesize that the accumulation of rab7⁺/lamp1⁺ vesicles around the SCV results from the inhibition of a downstream transport step as the consequence of the translocation into the cytoplasm of bacterial effectors. *Salmonella typhimurium* expresses type III secretion systems that direct the translocation of several bacterial proteins directly into the host cell (Kubori *et al.*, 1998). The presence of these effectors in the vicinity of the phagosome would explain the fact that rab7⁺/lamp1⁺ vesicles are observed in close contact with SCVs. Whether *S.typhimurium* produces a toxin capable of directly inducing this effect remains to be demonstrated.

The analogy between our observations and the cellular effects of VacA extends also to the role of rab7. This GTPase has been shown to play an essential role in the VacA-induced vacuolization of HeLa cells (Papini *et al.*, 1997). Interestingly, we observed a substantial increase in the size of clustered rab7⁺ vesicles in infected rab7-WT- or rab7-Q67L-HeLa cells (Figure 5). Conversely, the dominant-negative mutant of rab7 (T22N) prevents the accumulation of these vesicles (not shown). Similar opposite effects of dominant-positive or dominant-negative mutants of rab5 on the size of early endosomes have been observed (Stenmark *et al.*, 1994) and were attributed to the well-documented role of rab5 in the control of the homotypic fusion of early endocytic compartments (Gorvel

et al., 1991). Our results suggest that rab7 may be required for the homotypic fusion of rab7-containing vesicles.

In conclusion, the data presented herein suggest that *S.typhimurium* triggers the accumulation, in the proximity of its vacuole, of transport vesicles that connect late endosomes to lysosomes. We propose that rab7 regulates both the homotypic fusion activity of rab7- and lamp1-enriched vesicles and their heterotypic fusion with the SCV. The search for bacterial effectors of intracellular trafficking pathways and the identification of their targets is an exciting challenge and will undoubtedly lead to a better morphological and biochemical understanding of transport to lysosomes and other endocytic compartments.

Materials and methods

Antibodies

Human polyclonal antiserum against EEA1 was kindly provided by Dr Ban-Hock Toh (Monash Medical School, Melbourne, Australia). Mouse monoclonal antibody H68.4 specific to residues 3–28 of the human TfR cytoplasmic tail was from Zymed (San Francisco, CA). Rabbit antibodies against the 11-residue cytoplasmic domain of lamp1 and against the 33 C-terminal residues of rab7 have been described previously (Méresse *et al.*, 1997; Steele-Mortimer *et al.*, 1999). Mouse anti-human lamp2 was a generous gift of Dr Minoru Fukuda (The Burnam Institute, La Jolla Cancer Research Foundation, La Jolla, CA). Fluorescein isothiocyanate (FITC), Texas red, phycoerythrin (PE) and Cy5-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories (Immunotech, France).

Cells and cell culture

HeLa cells expressing myc-rab7-WT or myc-rab7-Q67L in a tetracycline-regulated manner have been described previously (Méresse *et al.*, 1995). HeLa cells expressing rab7-T22N in a tetracycline-regulated manner were obtained in a similar manner by co-transfecting HITA1 cells (Gossen and Bujard, 1992) with pUHDmyc-rab7-T22N and pSV2gpt. pUHDmyc-rab7-T22N was obtained by directed mutagenesis of the pUH rab7-WT as described elsewhere (Méresse *et al.*, 1995). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 0.1 µg/ml ATc at 37°C in 7% CO₂. ATc was added every other day. For subsequent experiments, induction of rab7 expression was obtained by trypsinizing and growing cells for 2 days in the absence of ATc. Medium was changed after 24 h. For immunofluorescence experiments, cells already grown for 2 days in the absence of ATc were trypsinized and seeded at a surface ratio of 1/10 in 10 cm dishes containing 12 mm glass coverslips and grown for 24 h. For flow cytometric analysis, cells were seeded at a surface ratio of 1/5 in 10 cm dishes and grown for 24 h.

Bacterial infection of HeLa cells

The *S.typhimurium* strain 1344 was used (Francis *et al.*, 1992). GFP-*S.typhimurium* was obtained by electroporating *S.typhimurium* with pBR322-GFPmut1 obtained from Dr Stanley Falkow (Stanford University School of Medicine, Stanford, CA). Bacteria were grown overnight at 37°C with shaking and then subcultured at a dilution of 1:33 in fresh Luria-Bertani broth and incubated at 37°C with shaking for 3 h. The bacterial inoculum was prepared by pelleting the culture at 10 000 r.p.m. in a tabletop centrifuge for 2 min and then directly resuspending the bacteria in phosphate-buffered saline (PBS). The inoculum was diluted in Earle's buffered salt solution (EBSS), pH 7.4, so that ~50 or 200 bacteria were added per cell for immunofluorescence and for flow cytometric analysis, respectively. HeLa cells were washed once with EBSS and 8 ml of EBSS containing the bacterial inoculum were added. The infection was carried out at 37°C for 5 or 10 min for immunofluorescence and for flow cytometric analysis, respectively. Cells were then washed extensively with PBS in order to remove free bacteria and either processed (5 and 10 min samples) or incubated further in growing medium supplemented with 50 µg/ml gentamicin at 37°C, 7% CO₂. Medium was removed at 1 h p.i. and replaced by growth medium supplemented with 10 µg/ml gentamicin. When indicated, 50 µM cycloheximide were added to cells 3 h before infection. Cycloheximide was then also present during the subsequent steps of infection.

Immunofluorescence

Cells grown on coverslips were washed rapidly with PBS, fixed with 3% formaldehyde in PBS, pH 7.4, for 15 min, washed extensively with 50 mM NH₄Cl in PBS and washed twice with 0.1% saponin in PBS. Cells were incubated for 20 min with primary antibodies diluted in 10% horse serum, 0.1% saponin in PBS, washed extensively with 0.1% saponin and incubated for 20 min with secondary antibodies. Coverslips were then washed, mounted in Mowiol and viewed under a Leica TCS 4DA confocal microscope. For double/triple staining experiments, identical optical sections are presented.

Flow cytometric analysis of subcellular particles

HeLa cells grown on 10 cm dishes were infected with GFP-S.typhimurium in EBSS for 10 min, washed extensively with PBS and either put on ice (10 min samples) or incubated further up to 110 min (2 h sample) as described above. All subsequent manipulations were performed on ice and buffers were chilled and pre-filtered (0.45 µM). Two 10 cm diameter dishes were used for each time point. Cells were washed extensively with PBS containing 0.2% bovine serum albumin (BSA) then scraped with a rubber policeman and gently homogenized in homogenization buffer (HB) containing 250 mM sucrose, 3 mM imidazole, pH 7.4, 0.1% gelatin, 0.5 mM EGTA and a protease inhibitor cocktail (1 µg/ml antipain, 1 µg/ml pepstatin, 1 µg/ml leupeptin, 15 µg/ml benzamidin). HB was added to the homogenate to give a final volume of 3 ml and a PNS was prepared by three successive centrifugations at 100 g for 5 min. For immunolabelling, 50 µl of PNS were gently mixed with 50 µl of an appropriate dilution of primary antibodies in PBS containing 5% normal horse serum and incubated for 10 min on ice. PE- and Cy5-conjugated secondary antibodies diluted in 50 µl of PBS were then added (1/300 final dilution) for 10 min on ice. Samples were diluted with 1 ml of PBS and immediately analysed by triple-colour flow cytometry using a Becton Dickinson FACStar Plus and CellQuest software (Becton Dickinson, Franklin Lakes, NJ). The filter settings were 530/30 nm, 575/26 nm and 660/13 nm band-pass for FITC (FL1), PE (FL2) and Cy5 (FL4), respectively. An analysis gate containing FITC-positive particles was set using the FL1-histogram and used for double and triple fluorescence quadrant analysis. For each point, a total of 2×10⁴ particles, of which 2×10³–4×10³ were SCVs, were analysed by flow cytometry. We determined both the percentage of SCVs containing the marker and the mean fluorescence of SCVs which reflect the relative abundance of a given epitope.

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