

The small GTP binding protein rab7 is essential for cellular vacuolation induced by *Helicobacter pylori* cytotoxin

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The VacA cytotoxin, produced by toxigenic strains of *Helicobacter pylori*, induces the formation of large vacuoles highly enriched in the small GTPase rab7. To probe the role of rab7 in vacuolization, HeLa cells were transfected with a series of rab mutants and exposed to VacA. Dominant-negative mutants of rab7 effectively prevented vacuolization, whereas homologous rab5 and rab9 mutants were only partially inhibitory or ineffective, respectively. Expression of wild-type or GTPase-deficient rab mutants synergized with VacA in inducing vacuolization. *In vitro* fusion of late endosomes was enhanced by active rab7 and inhibited by inactive rab7, consistent with vacuole formation by merging of late endosomes in a process that requires functional rab7. Taken together, the effects of overexpressed rab proteins described here indicate that continuous membrane flow along the endocytic pathway is necessary for vacuole growth.

Keywords: cytotoxin/gastric ulcer/*Helicobacter pylori*/late endosomes/rab7

Introduction

Strains of *Helicobacter pylori* producing cytotoxin VacA are involved in the development of atrophic gastritis, gastroduodenal ulcers and stomach adenocarcinoma in man (Warren and Marshall, 1983; Marshall *et al.*, 1985; Blaser, 1993; Eurogast Study Group, 1993; Parsonnet *et al.*, 1994; Telford *et al.*, 1994a). Oral administration of purified VacA to mice induced degeneration of the gastric mucosa and recruitment of inflammatory cells, two key events in the progression toward gastric ulcer (Blaser, 1993; Telford *et al.*, 1994b; Ghiara *et al.*, 1995). Strains of *H. pylori* with an altered *vacA* gene are non-cytotoxic (Phadnis *et al.*, 1994; Schmitt and Haas, 1994). These and other evidence indicate that VacA is a major virulence factor, directly involved in the pathogenesis of human gastroduodenal ulcers (Tompkins and Falkow, 1995).

The 140 kDa precursor of VacA is cleaved at the

C-terminal domain and released into the extracellular medium as a 95 kDa mature protein which oligomerizes into heptamers and hexamers (Cover *et al.*, 1994; Phadnis *et al.*, 1994; Schmitt and Haas, 1994; Telford *et al.*, 1994b; Lupetti *et al.*, 1996). In addition, VacA can be nicked by bacterial proteinases into two fragments with no change of biological activity (de Bernard *et al.*, 1995; Lupetti *et al.*, 1996). The C-terminal 58 kDa fragment is able to increase the permeability of liposomes to monovalent cations at low pH values (Moll *et al.*, 1995), similarly to several bacterial protein toxins with intracellular targets (Montecucco *et al.*, 1994). VacA is activated by a short exposure to low pH and becomes resistant to strong acidification and pepsin degradation, conditions which mimic the intragastric environment (de Bernard *et al.*, 1995).

The lumen of the large vacuoles induced by VacA in cultured cells (Cover and Blaser, 1992) as well as *in vivo* (Telford *et al.*, 1994b) is acidified by the proton pumping activity of a membrane-bound vacuolar-type ATPase (Cover *et al.*, 1993; Papini *et al.*, 1993a,b, 1996). The *H. pylori*-induced vacuoles contain fluid phase markers and their membrane is highly enriched in rab7 (Papini *et al.*, 1994), a small GTP binding protein previously shown to be associated with late endosomal compartments (Chavrier *et al.*, 1990). The identification of the molecular targets of bacterial toxins has contributed greatly to the present understanding of cell physiology. Very recently, clostridial neurotoxins were shown to block neuroexocytosis, a highly regulated vesicular trafficking process, by cleaving specifically three proteins involved in synaptic vesicle docking and fusion (Montecucco and Schiavo, 1995). It is expected that the elucidation of the mechanism of action of VacA will also contribute to the understanding of membrane traffic in eukaryotic cells.

Available evidence favours the possibility that vacuoles derive from late endosomes and that VacA cytotoxin alters membrane trafficking at this step (Papini *et al.*, 1994). Vacuoles appear as round structures of up to several micrometres in diameter, a size which can be reached only upon fusion of several smaller compartments. The toxin could promote endosome–endosome fusion either directly or through stimulation of a fusogenic protein. Alternatively, VacA could inhibit directly or indirectly the transport from late endosomes to lysosomes. The large amount of enrichment of rab7 on vacuolar membranes prompted us to investigate the role played by this small GTPase in the genesis and growth of vacuoles induced in cells by VacA.

Rab proteins are known to regulate the extent and the specificity of intracellular membrane traffic in eukaryotic cells (Novick and Brenwald, 1993; Simons and Zerial, 1993; Zerial and Stenmark, 1993; Nuoffer and Balch, 1994; Pfeffer, 1994). These proteins are anchored to

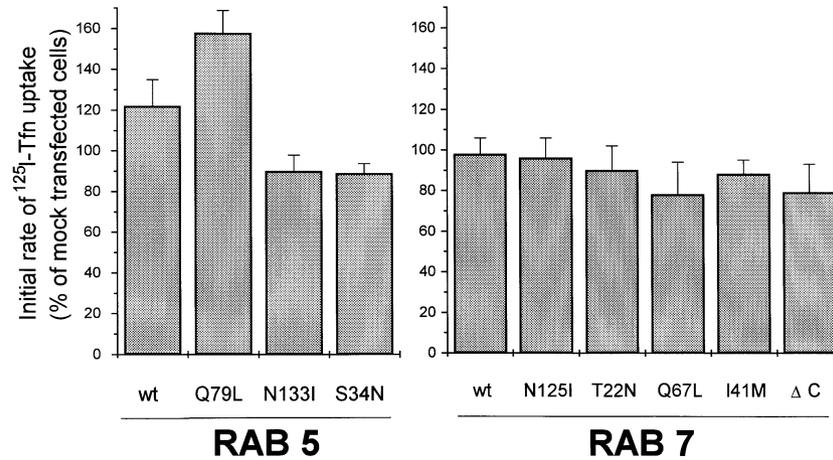


Fig. 1. Effect of overexpression of wild-type and mutated rab5 and rab7 on the rate of transferrin uptake by HeLa cells. Cells overexpressing the indicated rab proteins were incubated for 1 h at 4°C with [¹²⁵I]Tfn (100 ng/ml). After washing in the cold, the initial rate of Tfn endocytosis was determined as described in Materials and methods, corrected for transfection efficiency and was represented as a percentage of the value of mock-transfected cells. Values are the means of two experiments, and bars represent the range.

specific intracellular compartments by geranyl-geranylation at their C-termini (Magee and Newmann, 1992; Seabra *et al.*, 1992), and cycle between GDP-bound and GTP-bound forms (Simons and Zerial, 1993). Similarly to what was found for ras protein, point mutations in highly conserved regions can stabilize rab proteins in either the GDP- or the GTP-bound conformation and cause them to act as dominant interfering mutants. Inactive rab mutants are generated by substitution of Asn with Ile in the NKXD region, which results in low affinity binding of both GDP and GTP (Wolworth *et al.*, 1989; van der Sluijs *et al.*, 1991; Bucci *et al.*, 1992; Tisdale *et al.*, 1992), or substitution of Ser/Thr with Asn in the active site with a consequent high preference for GDP binding (Tisdale *et al.*, 1992; John *et al.*, 1993; Barbieri *et al.*, 1994; Nuoffer *et al.*, 1994; Pind *et al.*, 1994; Riederer *et al.*, 1994; Stenmark *et al.*, 1994). Rab mutants that are stabilized in the GTP-bound form are obtained by replacement of an active site Gln residue with Leu, which blocks intrinsic GTPase activity (Li and Stahl, 1993; Frech *et al.*, 1994; Stenmark *et al.*, 1994; Feng *et al.*, 1995; Meresse *et al.*, 1995), or by a Ile/Met substitution which compromises the interaction with GAP (GTPase-activating protein), a stimulator of GTP hydrolysis *in vivo* (Becker *et al.*, 1991).

Here we have overexpressed in HeLa cells wild-type and several mutants of three rab proteins associated with the endocytic pathway: rab5, which regulates endocytosis at early steps (Gorvel *et al.*, 1991; Bucci *et al.*, 1992; Stenmark *et al.*, 1994), rab7, implicated in transport from early to late endosomes (Feng *et al.*, 1995) or from late endosomes to lysosomes (Meresse *et al.*, 1995) and rab9, which regulates membrane cycling between late endosomes and the *trans*-Golgi network (TGN) (Lombardi *et al.*, 1993; Riederer *et al.*, 1994). After evaluation of their effect on transferrin (Tfn) uptake and epidermal growth factor (EGF) degradation and recycling, we assessed by morphological and biochemical means whether the presence of the various rab mutants modifies VacA effects. Rab7 was found to play an essential role in vacuole formation and the results suggest that VacA

induces an alteration of the balance between rab7-controlled homotypic fusion and fission of late endosomes.

Results

Expression of rab proteins in HeLa cells

Mutant and wild-type forms of rat rab5, rab7 and rab9 were transiently overexpressed in HeLa cells by infection with the recombinant vaccinia virus vT7, bearing the gene for phage T7 RNA polymerase. This was followed by transfection of pGEM plasmids, containing the rab gene behind the T7 promoter, induced by the cationic phospholipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Fuerst *et al.*, 1986; Stenmark *et al.*, 1995). The following rabs were transfected. The dominant-negative mutants were GDP-GTP low affinity mutants rab5 N133I, rab7 N125I and rab9 N124I, analogous to yeast Sec4 N133I (Walworth *et al.*, 1989); GDP binding mutants rab5 S34N, rab7 T22N and rab9 S21N, analogous to p21 ras S17N (Feig *et al.*, 1986; Ridley *et al.*, 1992). The GTPase-deficient mutants were rab5 Q79L and rab7 Q67L, analogous to p21 ras Q79L (Der *et al.*, 1986; Adari *et al.*, 1988; Tisdale *et al.*, 1992; Walworth *et al.*, 1992), and rab7 I41M which is defective in GAP interaction and analogous to a yeast Ypt1 mutant (Becker *et al.*, 1991). Rab7ΔC, a deletion mutant of rab7 lacking the C-terminal isoprenylation site, was included as a control. By Western blotting analysis with isotype-specific antibodies, we calculated that all the above-mentioned rab proteins were >30 times overexpressed in HeLa cells within 4 h of transfection with respect to their endogenous counterpart (not shown). A large fraction of cells (40–85%) were transfected, as demonstrated by immunofluorescence staining (not shown).

Effect of rab5 and rab7 mutants on the endocytic pathway

Uptake and recycling of Tfn and recycling and degradation of EGF are well-characterized indicators of the trafficking from the plasma membrane to early endosomes and from

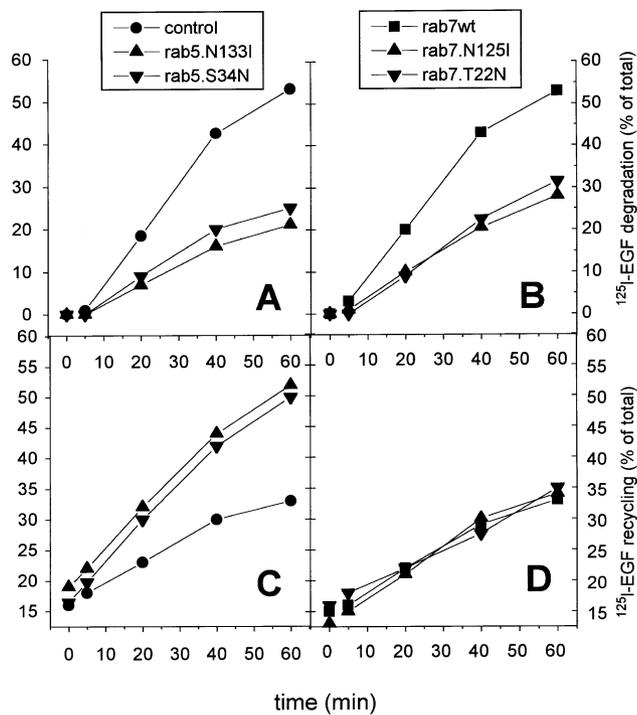


Fig. 2. Rate of EGF degradation and recycling by HeLa cells overexpressing dominant-negative mutants of rab5 and rab7. [^{125}I]EGF (50 ng/ml) was incubated for 10 min at 37°C with transfected or mock-transfected cells as specified, and chased at the same temperature after extensive washes. Aliquots of the extracellular medium were recovered at the indicated times and the amount of TCA (10% w/v)-soluble (A and B) and TCA-insoluble (C and D) radioactivity was determined. Data from a typical experiment are shown and reported as the percentage of total radioactivity associated with cells after the 10 min pulse.

late endosomes to lysosomes, respectively. Consistent with previous studies (Bucci *et al.*, 1992; Stenmark *et al.*, 1994), the initial rate of Tfn endocytosis, a reliable parameter of the extent of clathrin-mediated endocytosis, was increased significantly by overexpressed wild-type rab5 (+20%) and rab5 Q79L (+60%), whereas overexpression of GTP binding-defective rab5 mutants resulted in a weak inhibition (Figure 1). In contrast, overexpression of wild-type and mutant rab7 proteins did not alter Tfn endocytosis significantly, a result consistent with the localization of rab7 downstream of the compartment involved in Tfn uptake and recycling.

The rate of EGF digestion into trichloroacetic acid (TCA)-soluble small peptides monitors its transport to lysosomes and was measured in transfected HeLa cells after incubation with human [^{125}I]EGF for 10 min, a time sufficient to load primarily early endosomes. After a lag phase of 5 min (Figure 2A), TCA-soluble radioactivity was released constantly by HeLa cells into the extracellular medium, whereas only a minor fraction of internalized EGF was recycled (Figure 2C). EGF degradation was inhibited in cells expressing either rab5 N133I and rab5 S34N or rab7 N125I and rab7 T22N (Figure 2A and B). Remarkably, while in the case of rab5 mutants the inhibition of EGF degradation was paralleled by its recycling into the extracellular medium, in the case of rab7 mutants recycling remained as low as in control cells and non-degraded EGF

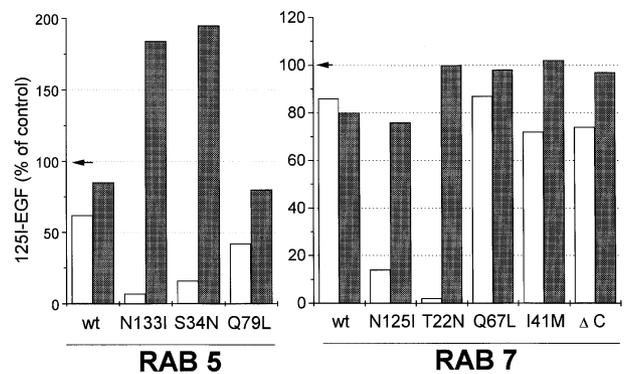


Fig. 3. EGF degradation and recycling by HeLa cells overexpressing wild-type and mutated rab proteins. Histograms summarize the effect of the indicated overexpressed rab proteins on the rate of EGF degradation and recycling. The velocity of TCA-soluble (white columns) or TCA-insoluble (grey columns) radioactivity discharge into the extracellular medium was determined as described in Figure 2, corrected for transfection efficiency and expressed as a percentage of mock-transfected cells.

accumulated intracellularly (Figure 2B and D). As shown in Figure 3, inhibition of EGF degradation by overexpression of GTP binding-defective rab5 and rab7 was almost complete, after correction for transfection efficiency. Wild-type rab5 and rab5 Q79L partially inhibited EGF degradation, without enhancing its recycling outside the cell. Overexpression of wild-type and GTPase-defective rab7 did not result in any significant change in EGF degradation or recycling.

Effect of overexpressing rab5, rab7 and rab9 mutants on intracellular vacuolation due to VacA

Transfected HeLa cells were treated with purified VacA for 2–3 h, a time period sufficient to obtain vacuolation of control cells. In a first set of experiments, vacuolation was determined morphologically, after indirect immunofluorescence staining to identify transfected cells. VacA induced the formation of perinuclear vacuoles, decorated with endogenous rab7, in HeLa cells overexpressing wild-type rab5 (Figure 4A–C). Overexpression of rab5 Q79L induced the formation of abnormally large early endosomes, as observed previously (Stenmark *et al.*, 1994). Such vacuoles are clearly distinguishable from those induced by VacA because they are smaller, mostly rab5-positive and rab7-negative (Figures 5 and 6). Moreover, as detailed later, vacuolation induced by rab5 Q79L did not result in an increased uptake of the acidotropic compound neutral red, probably because of the weak acidity of the early endosomal lumen (Mellman *et al.*, 1986). In these cells, VacA induced the formation of large vacuoles positive both for rab7 and rab5 (Figures 5 and 6). On the contrary, overexpression of rab5 N133I and rab5 S34N protected cells from VacA-induced vacuolation (Figure 7). In all fields examined, untransfected cells contained vacuoles, whereas cells expressing GTP binding-defective mutants did not exhibit large rab7-positive vacuoles.

HeLa cells overexpressing rab7 Q67L, rab7 I41M or wild-type rab7 were sensitive to VacA action and efficiently developed vacuoles upon exposure to the toxin (Figure 8A and B, and E and F). In cells expressing rab7 T22N and rab7 N125I, vacuoles did not form in the

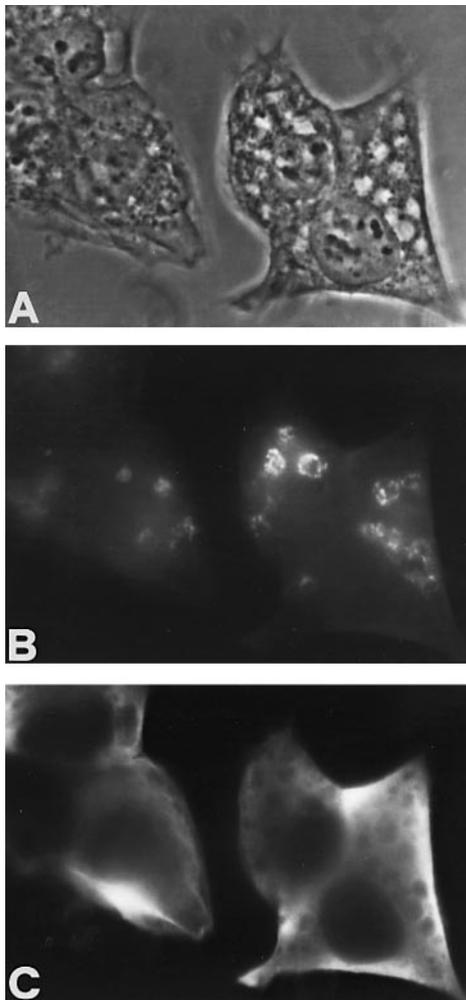


Fig. 4. Vacuolation of HeLa cells transfected with wild-type rab5 due to VacA. Cells expressing wild-type rab5 were treated with 0.1 μ M VacA for 3 h, fixed and double stained with specific antibodies to endogenous rab7 (**B**), or overexpressed exogenous rab5 (**C**) and FITC- or TRITC-conjugated secondary antibodies. (**A**) A phase contrast picture of the same cells is also shown. Magnification, $\times 500$.

presence of VacA, whereas in the same specimens untransfected cells were vacuolated (Figure 9). This protective effect of rab5 and rab7 mutants defective in GTP binding is specific. In fact, cells transfected with corresponding mutants of rab9, which regulates transport from late endosomes to the TGN (Lombardi *et al.*, 1993), behaved like untransfected cells (not shown). Vacuolated cells were $<5\%$ of transfected cells in samples overexpressing dominant-negative mutants of rab5 and rab7.

Results were quantitated by measuring the uptake of neutral red (NRU). The accumulation of this acidotropic compound in the lumen of vacuoles is a very reliable assay of VacA activity, depending on the number of vacuolated cells as well as on the number and dimensions of the vacuoles (Cover *et al.*, 1991; Papini *et al.*, 1993a). Figure 10 shows that dominant-negative mutants of rab7 strongly inhibited vacuolation due to VacA, which was lowered to $\sim 15\%$ of control, in agreement with the immunofluorescence microscopy analysis. Conversely, GTPase-defective mutants of rab7 slightly enhanced vacuole formation. No effect was observed when soluble rab7 Δ C was expressed, indicating that rab overexpression

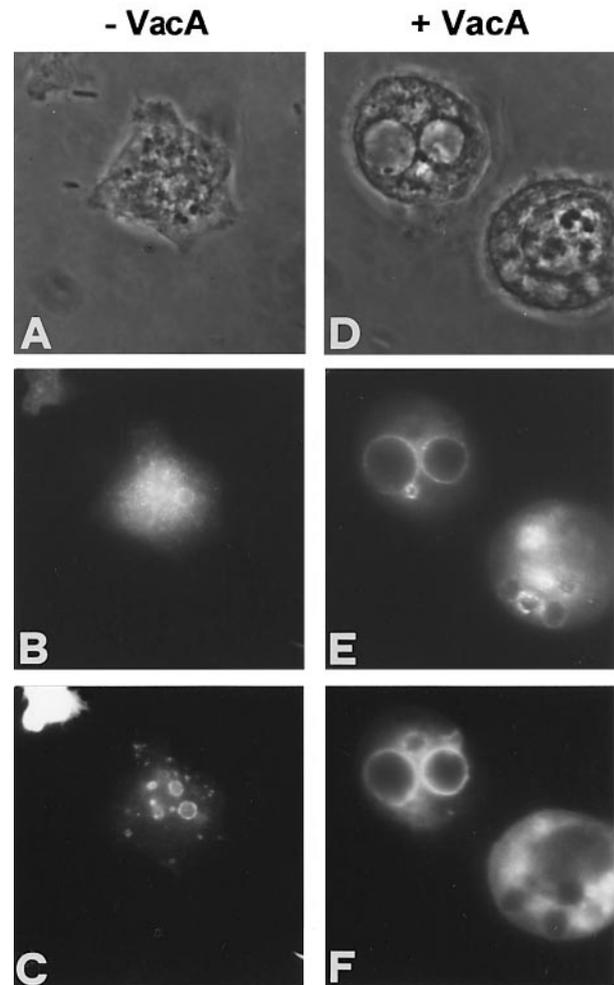


Fig. 5. Effect of rab5 Q79L on vacuolation induced by VacA. Untreated (**A–C**) or VacA-treated (**D–F**) HeLa cells overexpressing rab5 Q79L were stained to reveal endogenous rab7 (**B** and **E**) or rab5 Q79L (**C** and **F**) by indirect immunofluorescence. Magnification, $\times 500$.

per se does not cause vacuolation. Overexpression of dominant-negative mutants of rab5 was also found to inhibit VacA activity. In this case, however, inhibition determined by the NRU assay (45–52%) was less pronounced than that deduced by morphological evaluation. This difference may be explained by the smaller dimension of vacuoles. Overexpression of rab5 Q79L had a weakly promoting effect on vacuolation, while wild-type rab9 and dominant-negative mutants rab9 N124I and rab9 S21N had no effect.

Effect of rab7 and rab5 mutants on *in vitro* fusion between endosomes

The morphological and biochemical analyses indicate that functional rab7 is required for the process of vacuole formation induced by VacA *in vivo*. The observation that toxin-induced vacuoles share characteristics of late but not early endosomal compartments (Papini *et al.*, 1994) suggests that they may arise from the coalescence of rab7-positive late endocytic structures. As rab5 modulates homotypic fusion between early endosomes *in vitro* (Gorvel *et al.*, 1991), we tested whether rab7 can exhibit a similar effect on the fusion of late endosomes. HeLa

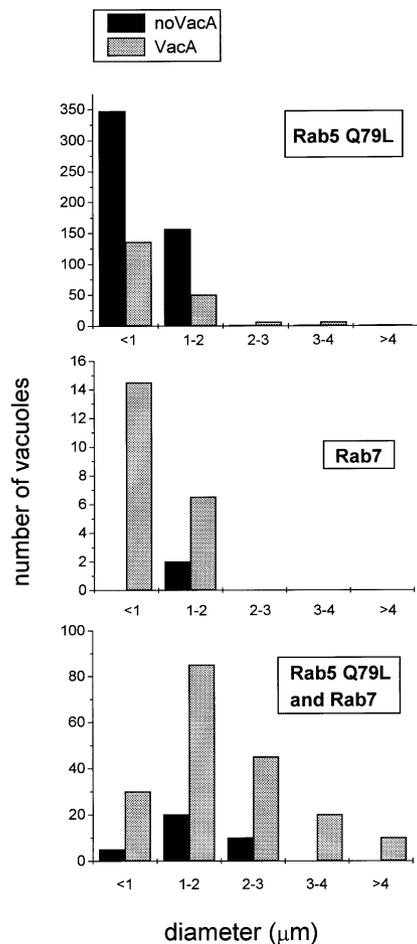


Fig. 6. Quantitative analysis of vacuolar structures induced by VacA in HeLa cells overexpressing rab5 Q79L. HeLa cells, transfected as in Figure 5 with rab5 Q79L and incubated or not with VacA, were double stained for rab5 Q79L and rab7. After random selection of several fields under a fluorescence microscope and collection of photomicrographs, vacuoles were counted and ranked according to the diameter size and the presence of rab5 Q79L, rab7 or both, as indicated. About 45 transfected cells were analysed for each condition.

cells were pulsed for 10 min with either biotinylated horseradish peroxidase (HRP) or avidin and incubated further for 25 min, to load prevalently late endosomes. Post-nuclear supernatants obtained from these cells were then mixed in the presence of homologous cytosol isolated either from untransfected cells or from cells overexpressing wild-type rab7, rab7 Q67L, rab7 T22N, rab5 Q79L and rab7 S34N. Endosome-endosome fusion, determined as HRP activity after detergent solubilization and immunoprecipitation (Gruenberg *et al.*, 1989), was 20–30% of maximal intermixing, and it was ATP dependent and *N*-ethylmaleimide (NEM) and GTP γ S sensitive (not shown). Addition of VacA did not affect fusion activity (not shown). As reported in Figure 11, fusion between late endosomes was increased slightly by cytosol containing wild-type rab7 and rab7 Q67L and partially inhibited by cytosol containing rab7 T22N, but was unaffected by cytosol containing either rab5 Q79L or rab5 S34N. Conversely, wild-type rab7 and mutants did not alter homotypic fusion between early endosomes, labelled by internalization of biotinylated HRP or avidin for 5 min

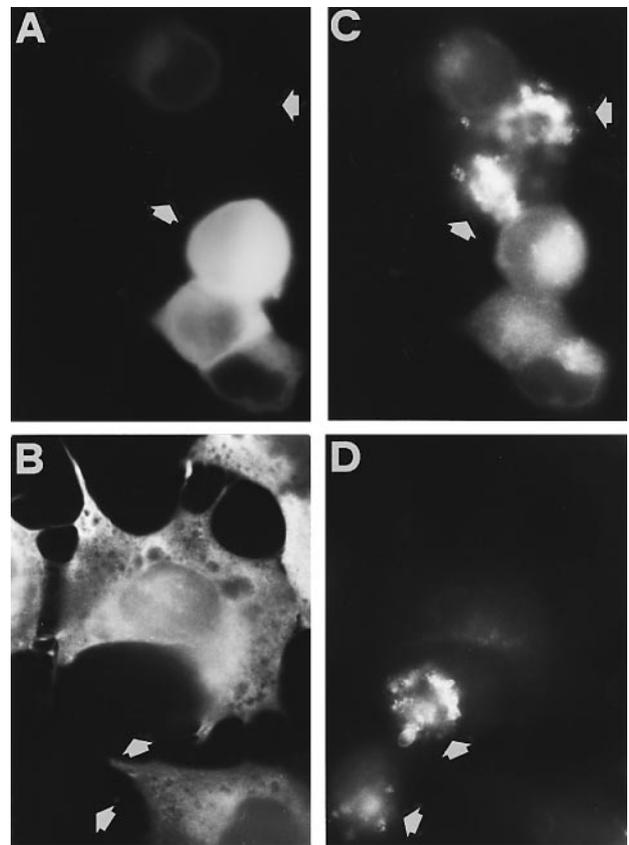


Fig. 7. Effect of rab5 S34N and rab5 N133I overexpression on VacA activity. Transfected HeLa cells were treated with 0.1 μ M VacA for 3 h and stained to detect rab5 S34N (A), rab5 N133I (B) and endogenous rab7 (C and D). The positions of vacuolated cells are indicated by arrows. Magnification 500 \times .

(not shown). Although we cannot dismiss the possibility that fusion events other than homotypic fusion take place to some extent in our *in vitro* assay, these data are consistent with the recent observation that late endosomes in HeLa cells overexpressing rab7 Q79L are larger than normal (Meresse *et al.*, 1995).

Discussion

The induction of numerous large intracellular vacuoles is the only known effect of the cytotoxin VacA of *H. pylori*, a major determinant of gastric ulcers (Leunk *et al.*, 1988; Blaser, 1993; Telford *et al.*, 1994b). The presence of V-ATPase and rab7 on the vacuole membrane and the inhibitory effect of microtubule-disrupting agents indicated that vacuoles could arise by coalescence of late endosomes (Papini *et al.*, 1994). In this study, we show that rab7 plays an essential role in the development of these vacuoles. Cells expressing rab7 mutants either defective in guanine nucleotide binding or stabilized in the GDP-bound form do not develop vacuoles when exposed to VacA. These rab7 mutants behave as dominant-negative with respect to the phenotype induced by the cytotoxin. Conversely, rab7 mutants stabilized in the GTP-bound active form have a slight but significant stimulatory effect on the same cellular response. It then appears that endogenous rab7 is hardly rate limiting in the vacuolation process. The effect of rab7 on vacuolation correlates with

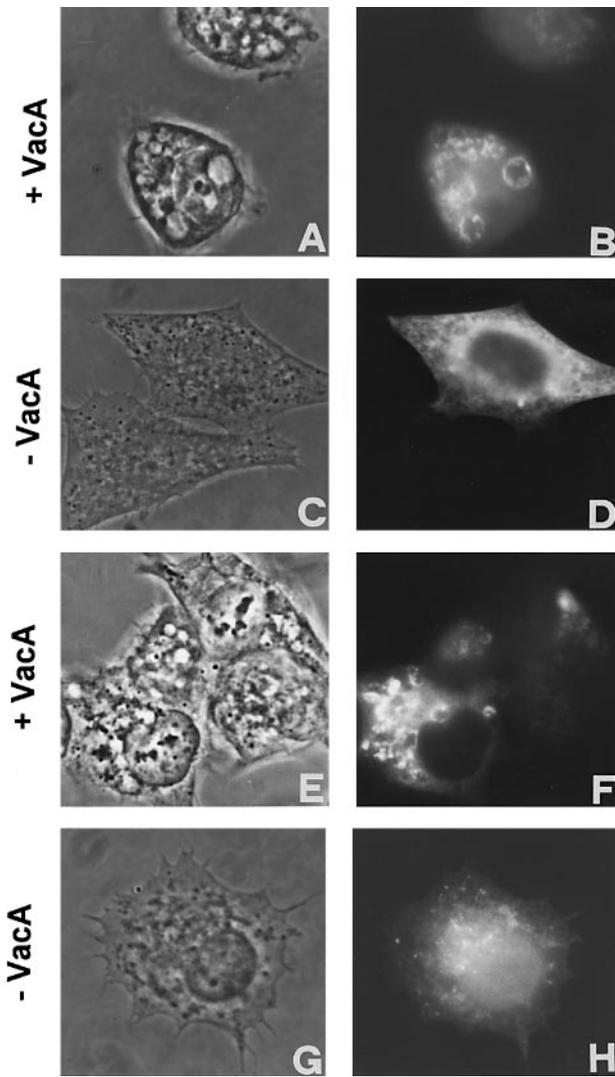


Fig. 8. Effect of rab7 Q67L and rab7 I41M overexpression on VacA activity. Cells expressing rab7 Q67L (A–D) or rab7 I41M (E–H) were treated with VacA (A, B, E and F) or with PBS (C, D, G and H) and stained with rab7-specific antibody to reveal overexpressed rab7 mutants. Magnification 350 \times .

that shown on late endosome fusion in a cell-free system. Cytosol from cells overexpressing positive rab7 mutants enhanced fusion between late endosomes, whereas cytosol derived from cells overexpressing negative rab7 mutants had an opposite effect. These rab7 mutants were inactive on the same assay performed on early endosomes. Taken together, these results suggest that vacuoles arise from late endosomes in a process that is controlled by rab7 in a positive way. The biogenesis of large vacuoles having a diameter in the micrometre range is likely to involve an increased membrane fusion activity. The rab5 and rab7 proteins appear to regulate endosome fusion sequentially, since we found no effect of rab7 on early endosome fusion as well as no effect of rab5 on late endosome fusion. An abnormally high fusion rate between late endosomes, supported by resident rab7, may be the crucial event underlying cellular vacuolation due to VacA.

The toxin is not fusogenic by itself, but this does not exclude the possibility that it may induce late endosome fusion indirectly by activation of an endogenous protein.

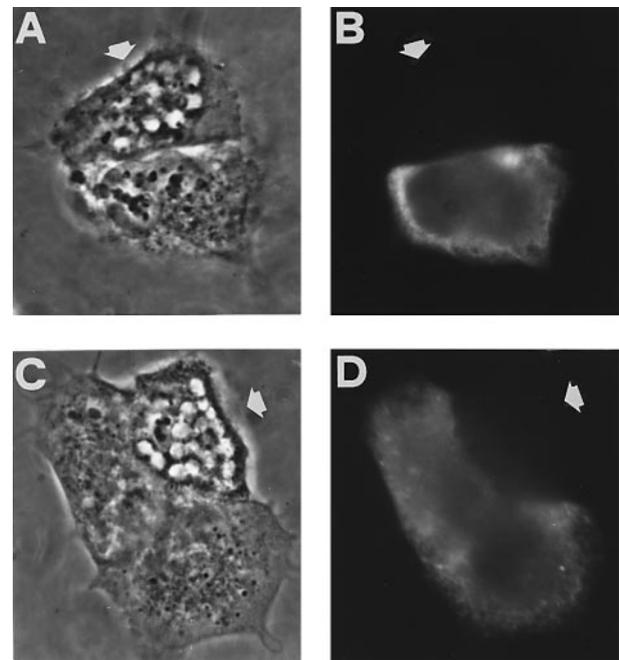


Fig. 9. Effect of dominant-negative mutants of rab7 on VacA activity. HeLa cells overexpressing rab7 T22N (A and B) and rab7 N125I (C and D) were treated with VacA (0.1 μ M) and immunostained to reveal rab7 mutants. The immunofluorescence staining allows transfected cells to be distinguished from untransfected cells. Since the overexpressed proteins accumulate unprenylated in the cytosol, the membrane staining is difficult to visualize. Arrows point to vacuolated cells. Magnification 500 \times .

The present results, however, indicate that a direct or indirect activation of rab7 by VacA is unlikely to be the sole mechanism underlying vacuole formation, since overexpression of GTPase-defective mutants of rab7 cannot mimic this process. It is not yet known which molecules besides rab7 can serve as a target for VacA.

Several rab proteins have been implicated previously in membrane trafficking in the endocytic pathway (Zerial and Stenmark, 1993), and our data support a role for rab7 at the late stages of transport. Rab5 regulates early endocytic trafficking since endocytosis of HRP and Tfn, as well as the overall organization of early endosomes, are deeply affected by overexpression of wild-type and mutant rab5 (Gorvel *et al.*, 1991; Bucci *et al.*, 1992; Stenmark *et al.*, 1994). Rab7 is involved in membrane transport between early endosomes and lysosomes (Feng *et al.*, 1995; Meresse *et al.*, 1995) and rab9 from late endosomes to the TGN (Lombardi *et al.*, 1993; Riederer *et al.*, 1994), respectively. Overexpression of dominant-negative mutants of rab5 (N133I and S34N) partially inhibits, though not completely, the formation of typical vacuoles by VacA. Conversely, overexpression of the GTPase-defective mutant rab5 Q79L is synergic to VacA. Since rab5 is absent from VacA-induced vacuoles (Papini *et al.*, 1994), these observations indicate that an efficient membrane flow from plasma membrane to late endosomes, mediated by early endosomes, is crucial for optimal vacuole formation. In fact, we found that sorting of EGF from early to late endosomes was blocked by rab5 N133I and rab5 S34N mutants and that, as a consequence, EGF was recycled back to the plasma membrane, similarly to Tfn (Figure 3A and C). It is conceivable that the extensive

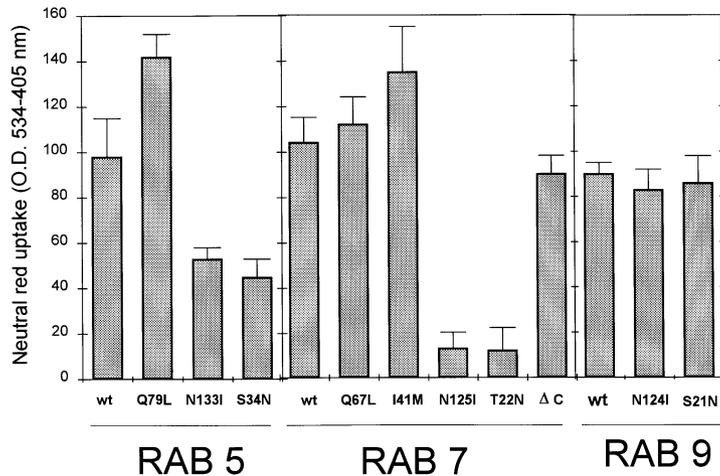


Fig. 10. Extent of vacuolation induced by VacA on HeLa cells overexpressing wild-type and mutated rab5, rab7 and rab9 as determined by neutral red uptake. HeLa cells expressing the indicated rab proteins were treated with 0.1 μ M VacA for 3 h and incubated for 8 min with 5 mM neutral red. The amount of vacuole-sequestered dye was determined, after extraction, as the optical density. After subtraction of background uptake due to cells transfected in the same way but treated with PBS, values were corrected for efficiency of transfection (determined in parallel by immunofluorescence staining) and reported as a percentage of the effect of VacA on mock-transfected cells. Data are the mean of 10 experiments, run in duplicate, and bars represent +SE.

fragmentation of early compartments induced by these mutants (Bucci *et al.*, 1992; Stenmark *et al.*, 1994) may prevent further membrane transport towards lysosomes either by inhibiting the proper formation of multivesicular carrier vesicles enriched in materials which are to be degraded (Gruenberg *et al.*, 1989; Aniento *et al.*, 1993) or by disrupting the vesicular-tubular network which mediates early endosomes' maturation into later compartments (Hopkins *et al.*, 1990; van Weert *et al.*, 1995), without affecting recycling to the plasma membrane. Vacuolation induced by VacA was slightly enhanced following wild-type rab5 or rab5 Q79L overexpression, in spite of the fact that transport to lysosomes was partially impaired, presumably due to massive coalescence of early endosomes. Compensation of the partial reduction of late endosome delivery by increased endocytosis may explain this apparent discrepancy. Alternatively, as suggested by double immunofluorescence staining (Figures 5 and 6), a higher degree of heterotypic fusion between rab5- and rab7-positive vacuoles induced by VacA may take place in these transfected cells.

In this study, we have shown that a transport step on the way to lysosomes, distal to early endosomes, is almost completely blocked by overexpression of GTP binding-defective mutants of rab7. In principle, this is in agreement with a role for rab7 in the transport either from early to late endosomes (Feng *et al.*, 1995), or from late endosomes to lysosomes (Meresse *et al.*, 1995). As long as membrane flow is considered, the first hypothesis appears more in keeping with the inhibitory effect of dominant-negative mutants of rab7 on vacuolization. In fact, an upstream blockade of membrane transport to late endosomes would be expected to inhibit vacuole formation, as observed. On the contrary, a blockade downstream of late endosomes is expected to help the formation of abnormally large late endosomes, in contrast to experimental evidence. However, the lack of membrane flow to late endosomes cannot account for complete inhibition of vacuolation, since a comparable block due to dominant-negative mutants of rab5 inhibited VacA action only partially.

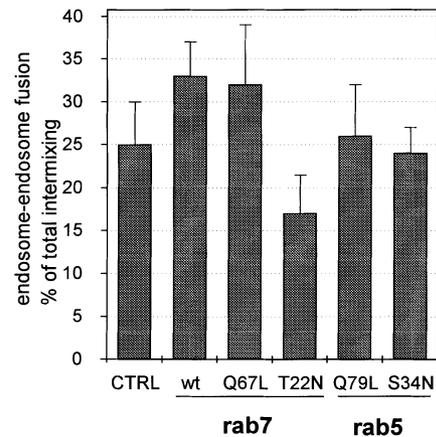


Fig. 11. Effect of rab7 and rab5 mutants on endosome-endosome fusion. HeLa cells were fed for 10 min with either avidin or biotinylated HRP and chased for 25 min. Fusion between late endosomes was then quantitated according to Gruenberg *et al.* (1989) after mixing post-nuclear supernatants from avidin- or biotinylated HRP-loaded cells in the presence of 3 mg/ml cytosolic proteins isolated from control HeLa cells or from HeLa cells overexpressing the indicated rab7 and rab5 proteins. Data, expressed as a percentage of total HRP activity, are the mean of four experiments run in duplicate. Bars represent +SE.

We propose, alternatively, that rab7-dependent regulation of homotypic fusion between late endosomes is of central importance to the biogenesis of VacA-induced vacuoles, as exemplified in Figure 12. Several lines of evidence support this hypothesis. Firstly, homotypic fusion during vacuolation is likely to occur, as suggested by the fact that rab7 membrane staining is not weakened but rather increased as vacuoles grow in size (Papini *et al.*, 1994). Secondly, rab7 is required for vacuolation. Thirdly, GTPase-defective rab7 mutants enhanced the fusion between late endocytic compartments *in vitro*, whereas dominant-negative mutants of rab7 substantially inhibit this process. This is consistent with the role played by Ypt7, the yeast homologue of rab7, in the formation of yeast vacuoles (Schimmoller and Riezman, 1993) and in

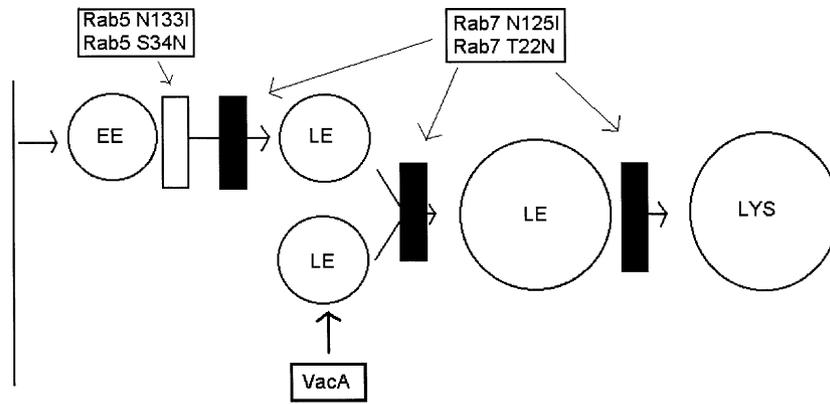


Fig. 12. Schematic representation of the steps of the endocytic pathway inhibited by rab5- and rab7-negative mutants and of the putative site of VacA action. Rab5-negative mutants block membrane sorting beyond early (or recycling) endosomes (empty bar), inducing the release of ligand otherwise delivered to lysosomes into the extracellular medium (see Figure 3). Rab7-negative mutants impair both membrane transport from early endosome to lysosomes at a site located downstream of early endosomes and, most likely, homotypic fusion between late (or pre-lysosomal) endosomes (black bars), (see Figures 3 and 11). The VacA cytotoxin from *H. pylori* is proposed to cause the formation of vacuoles which originate from a homotypic fusion between late endosomes, controlled by resident rab7. Membrane flow from early endosomes is clearly necessary for vacuole growth, as revealed by partial inhibition of vacuolization by dominant-negative mutants of rab5.

the homotypic fusion between vacuole precursors (Haas *et al.*, 1995).

In conclusion, the present data demonstrate, for the first time, a functional involvement of the endocytic pathway in vacuolar cell degeneration due to VacA. Efficient membrane flow from early to late endosomes and a functional rab7 are necessary for vacuole formation and growth. The present data suggest that rab7 is essential for the homotypic fusion of late endosomes, which appears to generate vacuoles and support their growth.

Materials and methods

Chemicals

DOTAP was from Boehringer. Pre-packed Quiagen columns were from Quiagen. IODO-GEN (1,3,4,6-tetrachloro-3,6-diphenylglycouryl) was from Sigma. Carrier-free Na^[125]I was from Amersham International.

Plasmids and transfection

DNAs encoding rab7, rab5a, rab5a Q79L, rab5a S34N and rab5a N133I inserted in pGEM-1 behind the T7 promoter have been described elsewhere (Chavrier *et al.*, 1990; Gorvel *et al.*, 1991; Stenmark *et al.*, 1994). Rab7 N125I, rab7 T22N, rab7 Q67L and rab7 I41M mutants were constructed by PCR-mediated mutagenesis (Landt *et al.*, 1990). In the first amplification, the following mutated oligonucleotides were used: 5'-CTGGAGTTGGTAAGAATCACTCATGAACCAG-3' for rab7 T22N; 5'-CAGAAAGTCTGCTCCCATTTGAGCTTTGTA-3' for rab7 I41M; 5'-GGAACCGTTCCAGGCCTGCTGTGTC-3' for rab7 Q67L and 5'-CTGTTTTCGAGGTCAATCTTGATTCCCAACACAACGAAAGGGA-3' for rab7 N125I. We used as outer primer for all amplifications oligonucleotides corresponding to the SP6 or T7 promoter present in the pGEM1 vector. The rab7ΔC mutant, which has a deletion of the last three amino acids of the C-terminus, was constructed using the mutated oligonucleotide 5'-TTAAGCTTTCAGCTTCCGCTGAGGCTTTG-3' and the T7 primer in a PCR amplification. The rab7ΔC oligonucleotide also has a *Hind*III site inserted to facilitate cloning. The resulting mutated bands from all amplifications were isolated by agarose gel electrophoresis, purified and cloned into pGEM1 under the control of the T7 promoter. The bands were then sequenced completely to ensure that the desired mutations were present and that no additional ones were introduced by the *Taq* polymerase. Wild-type rab9 and rab9 N124I, were from Dr Marino Zerial (EMBL, Germany), while rab9 S21N was a gift from Dr Suzanne Pfeffer (Stanford University School of Medicine, CA). Plasmids were purified by ion-exchange chromatography (Quiagen), spectrophotometrically quantitated and stored frozen (-70°C) in aliquots in Tris-HCl 10 mM, pH 7.4, Na-EDTA 1 mM. HeLa cells, seeded at a density of 30×10³/cm² 24 h previously, were infected with recombinant vaccinia

virus vT7 (Fuerst *et al.*, 1989) for 30 min, and transfected with plasmids containing *rab* genes behind the T7 promoter using DOTAP (Boehringer Mannheim) as described (Stenmark *et al.*, 1995). Mock-treated cells were infected with vT7 and treated with DOTAP without any plasmid.

Antibodies and proteins

Affinity-purified rabbit polyclonal antibodies to rab7 and rab9 were prepared as described (Chavrier *et al.*, 1990). Mouse monoclonal antibody to rab5 was from Dr M.Zerial (EMBL, Germany). Alkaline phosphatase-, TRITC- and FITC-conjugated antibodies to rabbit and mouse IgG were from Sigma. VacA was purified from an acellular culture filtrate of *H. pylori*, strain CCUG 17874, as already described (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 (de Bernard *et al.*, 1995). Human holotransferrin and recombinant human EGF (Sigma) were labelled with IODO-GEN as reported (Fraker and Speck, 1978). Specific activity was 0.5–0.8 μCi/μg.

Cell culture and intoxication

HeLa cells were cultured as monolayers in plastic flasks in Earle's modified minimal essential medium (MEM) containing 10% fetal calf serum (FCS) in a 5% CO₂ atmosphere at 37°C. At 4 h after transfection, cells overexpressing rab proteins or mock-transfected cells (treated with vT7 only) were treated with activated VacA (0.1 μM) in MEM, 10 mM hydroxyurea, 2% FCS and 5 mM NH₄Cl for 2–3 h at 37°C.

Measurement of Tfn uptake and recycling and of EGF degradation and recycling

Mock-treated or rab-transfected HeLa cells were incubated at 4°C for 1 h with 0.1 μg/ml of [¹²⁵I]Tfn in MEM without carbonate, plus 10 mM Na-HEPES, pH 7.4, 0.1% bovine serum albumin (BSA). After extensive washes at 4°C to remove unbound Tfn, cells were incubated with the same medium at 37°C. At 0, 2, 4, 6 and 25 min, extracellular, low pH-sensitive membrane-bound and intracellular radioactivity were determined (Hopkins and Towbridge, 1983).

Alternatively, cells were pulsed for 10 min at 37°C with 0.05 μg/ml of [¹²⁵I]EGF in MEM without carbonate, plus 10 mM Na-HEPES, pH 7.4, 0.1% BSA, rapidly washed and chased with the same medium at 37°C. After 0, 20, 40 and 60 min, TCA (10%)-soluble and TCA-insoluble radioactivity were determined in the extracellular medium with a Multi-Pris γ counter (Packard). Initial rates of Tfn endocytosis and of EGF degradation were corrected for transfection efficiencies, determined in parallel by immunofluorescence microscopy, and referred to control values of mock-treated cells. For each rab protein, >300 cells were counted randomly and the percentage of positive cells was always between 60 and 85%.

Preparation of HeLa cytosol containing overexpressed rab7 and rab5 proteins

Cytosol from eight Petri dishes (10 cm) of confluent HeLa cells was obtained after infection with vT7 and transfection with wild-type rab7,

rab7 Q67L, rab7 T22N, rab5 Q79L and rab5 S34N, using DOTAP, as described (Stenmark *et al.*, 1994). Rab-overexpressing 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, after centrifugation of post-nuclear supernatant (PNS) for 30 min at 46 500 r.p.m. with a 50Ti rotor (200 000 g), the supernatants were collected and either used fresh or frozen in liquid nitrogen. The relative amount of rab proteins in the cytosols was similar, as tested by Western blot followed by densitometry. Protein concentration, determined by the Bradford assay (Bradford, 1976), was 7–8 mg/ml.

Cell-free assay of endosome fusion

HeLa cells were incubated with either biotinylated HRP (2 mg/ml) or avidin (4 mg/ml) in IM (MEM, 10 mM Na-HEPES, 5 mM glucose, pH 7.4) for 5 min (early endosomes) or for 5 min followed by a 25 min chase (late endosomes) in IM. In this latter case, HRP activity co-localized with rab7 after fractionation on a 2–22% Ficoll velocity gradient in HB and was absent from the lysosomal fraction (not shown). PNS were mixed in the presence of 3 mg/ml cytosolic proteins from control cells or from cells overexpressing rab7, rab7 Q67L, rab7 T22N, rab5 Q79L and rab5 S34N in the presence of an ATP-regenerating system (2 mM ATP, 16 mM creatine phosphate and 80 µg/ml creatine phosphokinase) and 50 mg/ml 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 quantitated (Beaufay *et al.*, 1974) after immunoprecipitation of avidin–biotin–HRP complexes (Gruenberg *et al.*, 1989). In some control experiments, fusion was performed in the presence of an ATP-depleting system (hexokinase, 16 mM glucose), of 1 mM NEM or of 1 µM GTPγS. Total fusion was determined as HRP activity after immunoprecipitation in the absence of biotinylated insulin.

Indirect immunofluorescence microscopy

Cells were fixed with 3% paraformaldehyde for 20 min, treated with 0.27% NH₄Cl, 0.38% glycine for 10 min and permeabilized with 0.2% saponin, 0.5% BSA in PBS for 30 min. First, antibodies were diluted properly in the permeabilization medium in order to detect only over-expressed rab proteins, or, when indicated, in order to detect endogenous rab7, and incubated with cells for 1 h. After several washes, rhodaminated and fluoresceinated secondary antibodies were added for 30 min in the same medium and washed. Samples were mounted on 90% glycerol, 0.2% *N*-propylgallate in PBS and observed with a fluorescence microscope (Zeiss Axioplan, Jena, Germany).

Evaluation of vacuolation

Determination of the fraction of vacuolated cells. After VacA treatment, cells were fixed and immunostained to identify transfected cells. Within the same slide, fields were selected randomly and the number of transfected and untransfected cells showing clear large vacuoles was determined. At least 300 cells were counted per slide, and data were expressed as the percentage of untransfected cells. In one case, selected fields were photographed after indirect immunofluorescence double staining, and rab5 Q79L- and rab7-positive vacuoles were counted and ranked according to their diameter.

Determination of neutral red uptake (NRU). In samples run in parallel, cells were washed alternatively with PBS and 0.2% BSA, and incubated further for 8 min at room temperature with the same medium containing 5 mM neutral red. After washing of cells with PBS, neutral red was extracted with 70% ethanol and 0.37% HCl and the absorbance at 534 nm was determined. The increase in NRU, after subtraction of background values (those cells not affected by the toxin), corrected for the percentage of positive cells, was calculated with reference to mock-transfected cells, taken as 100%.

Western blot

Total cell or aliquots of cytosolic fractions were dissolved in 60 mM Tris-acetate, pH 6.8, 4% SDS, plus bromophenol blue, containing 0.1 mM phenylmethylsulfonyl fluoride, boiled for 2 min and run on 4–15% SDS-PAGE according to Laemmli (1970). Proteins were transferred to nitrocellulose for 2 h at 0.4 A in 25 mM Tris-HCl, 192 mM glycine, 5% methanol, pH 8.2. Rabs were revealed, after saturation of nitrocellulose overnight with 3% BSA, by incubating for 2 h with specific primary antibodies, and, after washes, with alkaline phosphatase-conjugated secondary antibody.

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