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Action Site and Cellular Effects of Cytotoxin VacA Produced by *Helicobacter pylori**

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ABSTRACT. Cells treated with the VacA toxin from *Helicobacter pylori* develop large membrane-bound vacuoles that originate from the late endocytotic pathway Using different experimental approaches, we showed that VacA can induce vacuoles by acting within the cell cytosol. Moreover, separation of VacA-induced vacuoles at an early stage of formation, using a novel isopycnic density ultracentrifugation method, allowed us to show that they resemble a hybrid compartment, containing elements of both late endosomes and lysosomes. Functional defects of the endocytotic pathway were also studied before any macroscopic vacuolation is evident VacA-intoxicated cells degrade extracellular ligands with reduced efficiency and, at the same time, they secrete acidic hydrolases into the extracellular medium, normally sorted to lysosomes. All these findings indicate that VacA translocates into the cell cytosol where it causes a lesion of the late endosomal/lysosomal compartments, such that protein trafficking across this crucial cross-point is altered with consequences that may be relevant to the pathogenesis of gastroduodenal ulcers

CONTENTS

1 INTRODUCTION

Chronic infection of stomach mucosa with toxigenic strains of *Helicobacter pylori* (Hp) is a major determinant in the pathogenesis of gastroduodenal ulcers and is associated with stomach carcinomas and lymphosarcomas (Warren and Marshall 1983; Marshall *et al.* 1985; *Eurogast Study Group* 1993; Parsonnet *et al.* 1994; Blaser 1993; Telford *et al.* 1994*a*; Censini *et al.* 1996). Isolation of toxin-producing strains from biopsies of gastroduodenal ulcers is common whereas mild gastritis is caused by nontoxigenic strains (Xiang *et al.* 1995). *H. pylori* strains with an altered VacA gene are noncytotoxic (Schmitt and Haas 1994; Phadnis *et al.* 1994). Moreover, orally administered VacA induces gastric mucosa degeneration and inflammatory cell recruitment (Marchetti *et al.* 1995; Ghiara *et al.* 1995). This toxin causes vacuolation of cells in culture (Leunk *et al.* 1988; Catrenich and Chestnut 1992) and therefore has been named VacA (Cover and Blaser 1992). Cell vacuoles are acidic and their membrane contains the vacuolar ATPase proton pump and the small GTP-binding protein Rab7: therefore, they were suggested to arise from late compartments of the endocytotic pathway (Papini *et al.* 1993, 1994, 1996; Cover *et al.* 1993).

VacA is produced as a 140-kDa precursor, 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, according to some authors (Telford *et al.* 1994*b*; Cover *et al.* 1994; Lupetti *et al.* 1996; Schmitt and Haas 1994; Phadnis *et al.* 1994), or into dodecamers, according to others (Cover *et al.* 1997). VacA shows the peculiar property of being a protein activated by, and resistant to, pH values that are reached in the stomach lumen (de Bernard *et al.* 1995). Recent observations show that low pH disassembles VacA oligomers into distinct 95-kDa subunits (Cover *et al.* 1997). Proteinases present in the Hp culture supernatants cleave VacA to generate two fragments of 37 and 58 kDa, which remain associated by noncovalent interactions (Telford *et al.* 1994*a*). Several features suggest that VacA may act as a A–B type toxin with the B part being responsible for cell binding and membrane translocation of the catalytic A subunit in the cytosol (Garner and Cover 1996; Moll *et al.* 1995; Montecucco *et al.* 1994). In agreement with this hypothesis, VacA should induce intracellular vacuoles by displaying its toxic activity in the cytosol compartments. This also implies that the target of the action of the catalytic domain of VacA is a cytosolic protein, or a membrane-bound protein facing the cytosol, involved in the regulation of membrane traffic at the late endosomal/lysosomal stage. This compartment is a crucial cross-way in cell membrane trafficking where proteins and other biological material deriv-

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ing from the cell surface and destined to degradation meet with acidic hydrolases coming from the *trans* Golgi network (TGN). There is good evidence that late endosomes and lysosomes are not the end compartments involved only in degradation (Lawrence and Brown 1982; Griffiths 1996*a*,*b*; Bright *et al.* 1997). Clear evidence in favor of this proposal is provided by the MHC-II antigen loading compartment which is a late endosomal/prelysosomal compartment and yet is able to move the antigen–MHC-II complex to the surface (Fernandez-Borja *et al.* 1995; Germain 1994; Watts 1997).

To obtain more information on the mechanism of action of VacA, we performed experiments aimed at the: (*a*) identification of the site of action of the toxin, (*b*) determination of the cellular alteration of protein delivery caused by vacuolization, and (*c*) isolation and biochemical characterization of vacuoles.

2 VacA IS ACTIVE IN THE CELL CYTOSOL

Microinjection of Hela cells with VacA results in intracellular vacuoles, indicating that it can act in the cell cytosol. To further prove this notion, and to overcome the difficulties inherent in the microinjection method, HeLa cells were also transfected with a plasmid bearing the genes encoding the 95-kDa toxin or the 140-kDa VacA precursor under the control of CMV promoter. A clear vacuolated phenotype developed after 24–30 h in transfected cells, in agreement with microinjection experiments. The percentage of transfected cells was further increased by transfection with a pGEM plasmid containing the VacA gene under the control of T7 promoter (pGEMp95), following infection with the recombinant vaccinia virus vT7, bearing the gene for phage T7 RNA polymerase (Fuerst *et al.* 1986), which we had used before to prove the requirement of rab7 for vacuolization to occur (Papini *et al.* 1997). The large majority of cells are transfected with this method and express the toxin in the cytosol already after 4 h, as demonstrated by indirect immunofluorescence staining with antibodies to VacA. Four hours after transfection vacuoles are clearly visible only in transfected cells. VacA was also transfected as a chimera with GFP, a green fluorescent protein that can be expressed in heterologous cells without loss of its intrinsic fluorescence (Chalfie *et al.* 1994; Heim *et al.* 1995) and that can be addressed to different cell organelles by tagging it with different signal sequences or by creating chimeras with cell proteins (Rizzuto *et al.* 1995, 1996). GFP mutants with improved fluorescence properties have been generated and here we have fused a bright GFP mutant, with Ser-65 mutated into Thr (Rizzuto *et al.* 1996), to the COOH terminal of 95-kDa portion of VacA. This chimeric gene was inserted into a pGEM plasmid and HeLa cells were transfected as described above. GFP tagging offers the advantage that cells do not need to be fixed and immunostained for VacA in order to localize the toxin. After 20 h from transfection, cells are vacuolated and contain a green fluorescence dispersed in the cytosol or adherent to the vacuolar membranes (de Bernard *et al.* 1997). Again, only fluorescent cells develop vacuoles.

Previous experiments have shown that GFP does not localize by itself to any particular cell region but that it is targeted by the protein fused to it (Rizzuto *et al.* 1995, 1996). Hence, the VacA-GFP chimera shows the same distribution obtained by immunofluorescence staining of cells expressing VacA. Taken together these results demonstrate that VacA can be expressed in eukaryotic cells and that it can induce cell vacuolization by acting in the cytosol (de Bernard *et al.* 1997).

3 ALTERATIONS OF PROTEIN TRAFFICKING AND INTRACELLULAR DEGRADATION IN VacA-TREATED CELLS BEFORE CELLULAR VACUOLATION

Acidic hydrolases are synthesized in the endoplasmic reticulum and reach the TGN, from which they are transported by clathrin-coated vesicles to endosomes and then to lysosomes (Kornfeld and Mellman 1989; von Figura and Hasilik 1986). Extracellular ligands to be degraded are endocytosed into early endosomes, move into late endosomes and eventually reach lysosomes. After entering endosomes, newly synthesized lysosomal hydrolases and molecules to be degraded are believed to share the same pathway *en route* to lysosomes (Trowbridge *et al.* 1994). To unmask possible functional alterations induced by VacA on late endosomes, the membrane traffic of newly synthesized cathepsin D and of epidermal growth factor (EGF) were studied. These two proteins are sorted to lysosomes from TGN and the cell surface, respectively. Cathepsin D, the major aspartyl proteinase in mammals, is carried to lysosomes *via* a M6P-dependent mechanism (Kornfeld and Mellman 1989; von Figura and Hasilik 1986). After synthesis as a proenzyme of 53 kDa in the endoplasmic reticulum, and addition of mannose 6-phosphate (M6P) to N-linked oligosaccharides, procathepsin D is transported to TGN and then to endosomes bound to M6P receptors. Here, acidic pH induces dissociation from its receptor and, after proteolysis which generates a 47-kDa intermediate form, cathepsin D reaches the lysosomes, where it is further proteolyzed to the mature form consisting of 31- and

14-kDa polypeptides (von Figura and Hasilik 1986; Trowbridge *et al.* 1994; Gieselman *et al.* 1983). A failure in the transport step beyond the TGN, either by blockade of the M6P-receptor cycling (Rijnboutt *et al.* 1992; Riederer *et al.* 1994) or by neutralization of late endosomes (Goda and Pfeffer 1988) leads to an increased release of newly synthesized procathepsin D into the extracellular medium through the constitutive secretion pathway. Thus, cathepsin D is a sensitive probe able to reveal defects of the late stages of the endocytotic pathway. The rate of generation of radiolabeled degradation products of ^{125}I-EGF is a reliable parameter of delivery to lysosomes (Trowbridge *et al.* 1994). In fact, after binding to specific receptors on the cell surface, EGF molecules are endocytosed, segregated from the recycling pathway in early endosomes and eventually carried to lysosomes where they are rapidly digested by acidic proteinases into small peptides, which are released to the extracellular medium.

To test the effect of VacA on procathepsin D maturation, HeLa cells were incubated with purified toxin and activated by preincubation at pH 2.0 as described before (de Bernard *et al.* 1995). After a few hours, when no sign of vacuolization was evident, cells were washed, sulfur starved, pulsed for 30 min with ^{35}S and chased for different time periods in the presence of M6P. Metabolically labeled cathepsin D was immunoprecipitated from the cell lysates and extracellular media with specific rabbit polyclonal antibodies. The relative amounts of procathepsin D (53 kDa), intermediate (47 kDa) and mature (31 kDa) forms of cathepsin D were determined after SDS-PAGE, autoradiography on X-ray films and densitometry. VacA inhibited the formation of both intermediate (47 kDa) and mature (31 kDa) cathepsin D. After a 2-h chase, the amount of mature cathepsin D was reduced to 40 % of control untreated cells, whereas the generation of the intermediate 47 kDa form dropped to about 60 %. Remarkably, such reduced formation of mature cathepsin D is associated with increased extracellular secretion of procathepsin D: the extracellular secretion and intracellular accumulation of procathepsin D account for the decrease of cathepsin D maturation. VacA acts at relatively low doses with an IC_{50} of about 40 nmol/L, and its preactivation by low pH is required for optimum inhibition of cathepsin D maturation (Satin *et al.* 1997). After exposure to VacA for 4 h, cells were incubated for 10 min with ^{125}I-EGF and its degradation was followed over time. The amount of ligand taken up in the first 10 min was the same in the control and in the VacA-treated cells, but the rate of ^{125}I-EGF degradation was decreased upon VacA treatment and this was accompanied by an intracellular accumulation of nondegraded EGF. The extent of recycling of intact EGF into the extracellular medium was unaffected by VacA treatment and this is consistent with an effect of VacA on a step along the endocytotic pathway after the early endosome stage (Satin *et al.* 1997).

Intracellular accumulation of procathepsin D and nondegraded EGF may be due, in principle, either to a blockade of their delivery to lysosomes, or to a decreased degradative capability. The first hypothesis implies that nonprocessed ligands accumulate in upstream compartments (TGN or late endosomes), whereas the second one implies their lysosomal accumulation. To discriminate between these two possibilities, homogenates from control or VacA treated cells were fractionated by ultracentrifugation on a Ficoll linear gradient (2–22 %, *W/V*), which allows the separation of slowly sedimenting organelles, such as early and late endosomes and Golgi membranes, from lysosomes. In intoxicated cells non-degraded ^{125}I-EGF colocalized with lysosomal fractions rather than with endosomes, thus suggesting that in VacA affected cells internalized EGF molecules can reach lysosomes, but are not digested efficiently there. After 30 min of metabolic labeling, procathepsin D is present exclusively in ER and Golgi. During the chase period, intermediate and mature forms are sequentially generated in control cells and, as expected, the mature form predominates in the lysosomal fractions. On the contrary, the relative proportions of nonprocessed or partially processed cathepsin D forms are higher in lysosomes of VacA-treated cells. The same experiment demonstrates directly that procathepsin D does not accumulate in endosomes. In contrast, after a 45-min chase the amount of procathepsin D in the Golgi-endosome fractions is much lower due to release to the extracellular medium, and the amount of total enzyme which reaches lysosomes does not vary significantly, after correction for intracellular retention of procathepsin D. These data indicate that procathepsin D and EGF are sorted to lysosomes in VacA-treated cells but that proper proteolysis does not occur (Satin *et al.* 1997).

4 VacA PARTIALLY NEUTRALIZES ENDO-LYSOSOMAL pH BEFORE INTRACELLULAR VACUOLATION

Low pH activates acidic proteinases responsible for procathepsin D partial cleavage and for the complete degradation of EGF and causes dissociation of hydrolases from M6P receptors inside late endosomes (Kornfeld and Mellman 1989; von Figura and Hasilik 1986; Trowbridge *et al.* 1994). To test the possibility that VacA affects the pH of endosomes and lysosomes, control and intoxicated cells were incubated with concentrated FITC-dextran for 1 h in order to load the whole endocytotic pathway, washed, and chased for

15 min to clear early endosomes and thus fluorescently label late endosomes and lysosomes. FITC-dextran was largely present in the perinuclear region of both control and intoxicated cells to a similar extent. Analysis of single cell fluorescence allowed us to determine the luminal pH of FITC-dextran-loaded compartments. The mean pH value of late endosomes and lysosomes of several cells was estimated to be about 5.65, to be compared with the value of about 5.25 of control cells. Such a variation is only slightly lower than that induced by 5 mmol/L NH_4Cl, which brings endo-lysosomal pH to 5.8. Moreover the increased pH value caused by VacA is also significant when compared with maximum neutralization obtained with 20 μmol/L monensin (pH 6.2). Thus, the increased endosomal/lysosomal pH, caused by VacA can account for the inhibition of the proteolytic activity of these compartments and for the decreased procathepsin D cellular retention (Satin *et al.* 1997).

5 VACUOLES INDUCED BY VacA ARE COMPARTMENTS INTERMEDIATE BETWEEN LATE ENDOSOMES AND LYSOSOMES

Analysis of the early events of cellular intoxication and an accurate mapping of the main vacuolar components will help in determining the intracellular target(s) of VacA and in understanding the mechanism of cellular intoxication by this bacterial toxin. BHK cells exposed to low VacA concentrations were fractionated with a novel, isopycnic density ultracentrifugation method, optimized for the clear-cut separation of late endosomes (and vacuoles that form upon VacA treatment) from lysosomes (Reaves *et al.* 1996; Molinari *et al.* 1997). Under these conditions, VacA causes pathological changes in compartments of the endocytotic pathway before alterations detectable by phase-contrast microscopy become apparent. In fact, we could follow the redistribution of Lgp110, a lysosomal membrane glycoprotein, to a compartment lighter than lysosomes, which also contains Rab7. This novel compartment is acidic and therefore swells when osmotically active weak bases are present, thus giving rise to the large vacuoles previously associated with VacA-activity. Parallel immunofluorescence staining allowed us to confirm that vacuoles are enriched in lysosomal membrane markers (Lgp110), contain low levels of lysosomal hydrolytic activities, are characterized by the presence of the late endocytotic marker Rab7, but are devoid of the cation-independent mannose-6-phosphate receptor (CI-M6PR; Reaves *et al.* 1996). These results indicate that VacA may induce the pathological accumulation of a mixed endo-lysosomal compartment. Such features resemble those of the compartment of antigen-presenting cells (APC) where antigens are processed and loaded on MHC-II molecules (Fernandez-Borja *et al.* 1995; Germain 1994). This raises the interesting possibility that VacA interferes in antigen processing and presentation by APC, thus preventing T-cell proliferation, an effect that would lower the immune response at the level of the stomach mucosa, allowing its colonization by pathogenic strains of *H. pylori*. The inhibition of antigen presentation is also expected to be hampered by the partial neutralization of endosomes described above, which may affect both antigen unfolding and processing. Interestingly, a redistribution of lysosomal membrane glycoproteins (Lgp110 and Lgp120) similar to the one obtained by VacA, was recently described as a consequence of the treatment of NRK cells with wortmannin, an inhibitor of PI-3 kinase (Reaves *et al.* 1996) which causes the appearance of two swollen late endocytotic compartments, one Lgp^+/CI-$M6PR^-$ with characteristic similar to the VacA-induced vacuoles, and one positive for M6PR that was not found in VacA-treated cells. The former compartment arises in response to the wortmannin-induced inhibition of the reformation of electron dense lysosomes from a late endosome/lysosome hybrid compartment. Similarly, it is conceivable that treatment with VacA inhibits membrane recycling and lysosomal reformation from such a hybrid compartment, that may still fuse with more lysosomes and late endosomes and therefore swell, particularly in the presence of membrane permeable amines. These analogies suggest the possibility that PI-3 kinase is implicated in the mechanism of VacA intoxication (Molinari *et al.* 1997).

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