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Effect of *Helicobacter pylori* Vacuolating Toxin on Maturation and Extracellular Release of Procathepsin D and on Epidermal Growth Factor Degradation*

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The effect of vacuolating toxin (VacA) from *Helicobacter pylori* on endosomal and lysosomal functions was studied by following procathepsin D maturation and epidermal growth factor (EGF) degradation in HeLa cells exposed to the toxin. VacA inhibited the conversion of procathepsin D (53 kDa) into both the intermediate (47 kDa) and the mature (31 kDa) form. Nonprocessed cathepsin D was partly retained inside cells and partly secreted in the extracellular medium via the constitutive secretion pathway. Intracellular degradation of EGF was also inhibited by VacA with a similar dose-response curve. VacA did not alter endocytosis, cell surface recycling, and retrograde transport from plasma membrane to *trans*-Golgi network and endoplasmic reticulum, as estimated by using transferrin, diphtheria toxin, and ricin as tracers. Subcellular fractionation of intoxicated cells showed that procathepsin D and non-degraded EGF accumulate in lysosomes. Measurements of intracellular acidification with fluorescein isothiocyanate-dextran revealed a partial neutralization of the lumen of endosomes and lysosomes, sufficient to account for both mistargeting of procathepsin D outside the cell and the decreased activity of lysosomal proteases.

The development of human upper gastroduodenal diseases such as atrophic gastritis, ulcer, stomach adenocarcinoma, and lymphoma has been recently ascribed to prolonged local infection with the Gram-negative bacterium *Helicobacter pylori* (1–5). Functional vacuolating toxin (VacA)[1] is produced by more aggressive parasite strains and is therefore regarded as a major pathogenic factor (6–11). Accordingly, experimental infection of animals proved that only VacA-producing *H. pylori* strains elicit gastric inflammation (12), and oral administration of purified VacA to mice induces epithelial degeneration of the stomach epithelium (13).

VacA is released as a 95-kDa protein that has a strong tendency to oligomerize in heptamers (14). Similar to bacterial protein toxins with intracellular targets, VacA is split by limited proteolysis at a specific site into two fragments, 37 and 58 kDa, which remain associated by noncovalent forces (15). Moreover, the recombinant 58-kDa fragment renders liposomes permeable to potassium at acidic pH (16). VacA induces in cultured cells the formation of perinuclear vacuoles that grow in size and gradually fill all the cytosol (6, 17, 18). Exposure of VacA to low pH induces the transition to a stable acidic form endowed with a higher vacuolating activity (19).

Previous studies have shown that vacuoles induced by VacA originate from membrane-bound compartments of the endocytic pathway, characterized by the presence of the V-ATPase and the small GTP-binding protein rab7 (20, 21). Complete blockade of V-ATPase by specific inhibitors (21–24) and suppression of rab7 functions by overexpressing homologous dominant negative mutants (25) demonstrated that these two molecules are essential to vacuole biogenesis. The V-ATPase may drive the accumulation of osmotically active, membrane permeant, weak bases into the vacuolar lumen (21), while Rab7 may be implicated in membrane fusion events, similarly to rab5 in the case of swollen early endosomal compartments (26). Based on this evidence, we suggested that VacA may interfere with normal membrane traffic at the level or in the close vicinity of late endosomes (25). Such compartments, also referred to as prelysosomal compartments, are crucial crossroads in the complex network of intracellular membrane traffic inside eukariotic cells (27). In fact, they sort proteins and lipids derived from the biosynthetic or the endocytic pathways and directed to lysosomes. Typically, acidic hydrolases synthesized in the endoplasmic reticulum reach the TGN where they are transported by clathrin-coated vesicles to endosomes and then to lysosomes (27, 28). 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 doomed to degradation are believed to share the same pathway *en route* to lysosomes (29). Such a picture is complicated by the presence of well demonstrated or putative recycling pathways, which are also supposed to determine the rate of protein transport to lysosomes (27).

In the present study, to unmask possible functional alterations induced by VacA on late endosomes, we investigated the membrane traffic of newly synthesized cathepsin D and of epidermal growth factor, two well characterized proteins which are sorted to lysosomes from TGN and the cell surface, respec-

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[1] The abbreviations used are: VacA, vacuolating toxin; EGF, epidermal growth factor; M6P, mannose 6-phosphate; BSA, bovine serum albumin; Tfn, transferrin; DT, diphtheria toxin; TGN, *trans*-Golgi network; TPCK, L-1-tosylamino-2-phenylethyl chloromethyl ketone; PBS, phosphate-buffered saline; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis.

tively. Cathepsin D, the major aspartyl protease in mammalians, is carried to lysosomes via a mannose 6-phosphate (M6P)-dependent mechanism (27). After synthesis as a proenzyme of 53 kDa in the endoplasmic reticulum, and addition of M6P to *N*-linked oligosaccarides (30), 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 proteolysed to the mature form consisting of 31- and 14-kDa polypeptydes (31–34). A failure in the transport step beyond the TGN, either by blockade of M6P-receptor cycling (35–37) or by neutralization of late endosomes (38–41) results in an augmented release of newly synthesized procathepsin D into the extracellular medium through the constitutive secretion pathway. Thus, cathepsin D is a sensitive indicator of defects of the late stages of the endocytic pathway.

The rate of generation of radiolabeled degradation products of ^{125}I-EGF is a reliable parameter of delivery to lysosomes (42, 43). 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 (29), where they are rapidly digested by acidic proteases into small peptides, which are released in the extracellular medium.

MATERIALS AND METHODS

Reagents—IODO-GEN (1,3,4,6-tetrachloro-3,6-diphenylglycouryl) was from Sigma. Carrier-free Na[^{125}I], L-[U-^{14}C]leucine (342 Ci/mol), and Tran^{35}S-label (1274 Ci/mmol) were from Amersham International (Amersham, UK). VacA was purified from acellular culture filtrate of *H. pylori*, strain CCUG 17874, as described previously (44), filter-sterilized, and stored at 4 °C in 50 mM NaP_i, pH 7.4, 145 mM NaCl (PBS). When necessary, VacA was activated by acid pH treatment immediately before use (19). Monomeric form of diphtheria toxin (DT) was purified as described previously (45), nicked by trypsin-L-1-tosylamino-2-phenylethyl chloromethyl ketone (TPCK) (46), and frozen in liquid nitrogen in 10 mM K-Hepes, 0.1 mM EDTA, pH 7.0, at the concentration of 2 mg/ml. Purified ricin was a kind gift from Dr. Marco Colombatti (University of Verona, Italy). Human holotransferrin (Tfn) and recombinant human epidermal growth factor (Sigma) were labeled with IODO-GEN (Sigma) as reported elsewhere (47). Specific activity was 0.5–0.8 μCi/μg. TPCK-treated trypsin (61 units/mg) was from Serva. Fetal calf serum (FCS) was from Flow (UK). Monensin, leupeptin, pepstatin A, benzamidine, aprotinin (24 trypsin inhibitory units/ml), TPCK, phenylmethylsulfonyl fluoride, FITC-dextran, and protein A-Sepharose were from Sigma. Monoclonal antibody to the cytosolic domain of human transferrin receptor and affinity-purified rabbit polyclonal antibodies to rat rab7 were provided by Dr. C. R. Hopkins (University College London, London, UK) and by Dr. M. Zerial (European Molecular Biology Laboratory, Heidelberg, Germany), respectively. Polyclonal rabbit antibody to human cathepsin D was from DAKO.

Cell Culture and Intoxication—HeLa cells were cultured as monolayer in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS in a 5% CO_2 atmosphere at 37 °C. Cells were suspended by trypsin-EDTA treatment and seeded in standard 24- or 6-well titer plates at the density of 40,000/cm^2 in growth medium 14–18 h before experiments. Cells were washed with DMEM, 2% FCS and further incubated at 37 °C with 5–200 nM purified VacA, preactivated or not by low pH treatment as described previously, in the same medium in 5% CO_2 for 4 h. In some experiments, after VacA intoxication as above, cells were washed with DMEM, 2% FCS and further incubated with 10^{-8} M DT or 10^{-7} M ricin, and after different times, the rate of protein synthesis inhibition was determined.

Uptake, Degradation, and Recycling of Tfn and EGF—Control or VacA-treated HeLa cells were incubated at 4 °C for 1 h with 0.1 μg/ml ^{125}I-Tfn in DMEM without carbonate, plus 10 mM Hepes-Na, pH 7.4, 0.2% BSA. After extensive washes at 4 °C, to remove unbound Tfn, cells were incubated with the same medium plus 2% FCS at 37 °C. After different times, extracellular, membrane-bound, and intracellular radioactivity was determined as described previously (48).

Alternatively, cells were pulsed for 10 min at 37 °C with 0.05 μg/ml ^{125}I-EGF in DMEM without carbonate, plus 10 mM Hepes-Na, pH 7.4, 0.2% BSA, rapidly washed, and chased with the same medium plus 2% FCS at 37 °C. After different times, trichloroacetic acid (10%)-soluble and -insoluble radioactivity was determined in the extracellular medium with a Multi-Prias γ-counter (Packard). In some experiments, nondegraded EGF was precipitated with trichloroacetic acid from fractions obtained from a 2 to 22% w/v Ficoll gradient.

Determination of Protein Synthesis Inhibition—Cells were rapidly washed with leucine-free Dulbecco's modified eagle's medium without $NaHCO_3$ and containing 10 mM Hepes-Na, pH 7.4, and further incubated with the same medium supplemented with 200 nCi/ml [^{14}C]leucine. After 4 min, trichloroacetic acid was added to each well (final concentration, 6%), and the precipitated radioactivity was measured by liquid scintillation. The rate of protein synthesis of DT and ricin-treated cells was calculated as the percentage of control cells (49).

Sorting and Processing of Cathepsin D—VacA-intoxicated or control cells were washed, sulfur-starved in sulfur-free medium for 30 min, pulsed with the same medium containing 50 μCi/ml Tran^{35}S-label for 30 min, washed with DMEM containing 2% FCS, 10 mM cysteine, and 5 mM M6P and further incubated in the same medium at 37 °C. At the desired time, extracellular medium was recovered, supplemented with a protease inhibitor mixture, and put in ice, while cells were washed with ice-cold PBS and dissolved in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM NaEDTA, 1% Triton X-100, pH 7.4), supplemented with protease inhibitors. Extracellular medium and cell lysates were spun at 14,000 rpm for 20 min at 4 °C, and the supernatants were incubated with anti-human cathepsin D rabbit antiserum (DAKO) (0.25% v/v) for 2 h at 4 °C. Protein A-Sepharose, previously equilibrated in lysis buffer, was added to samples, and after a further 18-h incubation with tumbling at 4 °C, immunocomplexes were recovered by centrifugation, washed five times with 50 mM Tris, 150 mM NaCl, 1 mM NaEDTA, pH 7.4, 1% deoxycholate, 0.1% SDS, once with 10 mM Tris-Cl, pH 7.0, and then dissolved in 62 mM Tris acetate, pH 6.8, 4% SDS. Samples were boiled for 2 min and run on 10% polyacrylamide gel electrophoresis according to Laemmli (51). Gels were dried and exposed to x-ray films, and the amount of the different forms of cathepsin D was determined after developing by densitometry. Data are expressed as percent of total signal. In some experiments, cells were homogenized after pulse and chase as above and fractionated on a 2 to 22% (w/v) Ficoll gradient, and cathepsin D was immunoisolated from fractions diluted with an equal volume of double-concentrated lysis buffer plus protease inhibitors.

Subcellular Fractionation—Control and VacA-treated cells, either pulse-chased with Tran^{35}S-label or treated with ^{125}I-EGF and further incubated for 40 min, were washed with ice-cold PBS, scraped in 0.25 M sucrose, 10 mM Hepes-Na, pH 7.4. Homogenization was obtained by passing the cell suspension 10 times up and down through a 1-ml blue tip on a pipette (Gilson Co. Inc., Worthington, OH) and 10 times through a 22G1 1/4 needle fitted on a 1-ml plastic syringe. Cell homogenate was centrifuged for 15 min at 3500 rpm, and supernatants (1 ml) were loaded on top of 2 to 22% w/v Ficoll-400 linear gradients (13 ml) made in UltraClear Beckmann centrifuge tubes. After centrifugation for 65 min at 35000 rpm in a SW40 Ti rotors, fractions of 1 ml were collected from the top and put in ice for further analysis.

Determination of Endosomal/Lysosomal pH—Cells were seeded on glass coverslips, and after treatment with 200 nM VacA as specified above, they were incubated for 60 min at 37 °C with DMEM, 20 mM Hepes, pH 7.4, 0.2% BSA containing 10 mg/ml FITC-dextran, washed, and further incubated for 15 min in the same medium with no FITC-dextran. Coverslips were mounted in a thermostatted chamber (37 °C), overlaid with 0.5 ml of PBS, 5.6 mM glucose and placed in the stage of an inverted microscope. Single cell fluorescence values were determined with excitation wavelengths of 494 and 450 nm and a fluorescein emission filter, with the aid of a computerized image analysis system. pH values were obtained by comparing the ratio of the two wavelengths fluorescence values, corrected for cell intrinsic fluorescence, with those of FITC-dextran dissolved in internalization media of different pH values (50).

Western Blot and β-N-Acetylglucosaminidase Activity—Total cell or aliquots of cytosolic fractions were dissolved in 60 mM Tris acetate, pH 6.8, 4% SDS, plus bromphenol blue, containing 0.1 mM phenylmethylsulfonyl fluoride, boiled for 2 min, and run on 4–15% SDS-PAGE according to Laemmli (51). Proteins were transferred on nitrocellulose for 2 h at 0.4 A in Tris/Cl 25 mM, glycine 192 mM, 5% methanol, pH 8.2. Rab7 and transferrin receptor 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. β-*N*-Acetylglucosaminidase activity was measured as described in Beafay *et al.* (52).

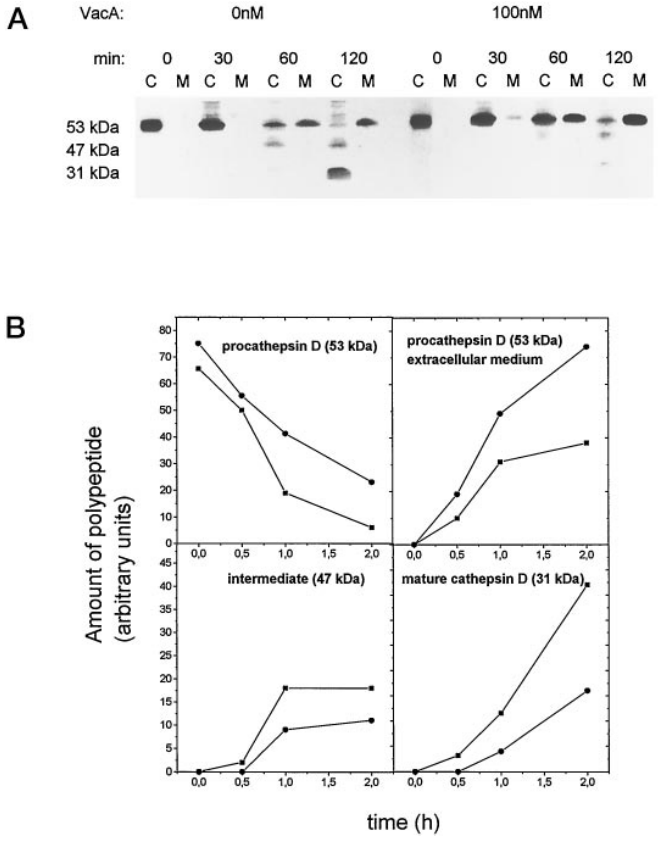

FIG. 1. **Effect of VacA on the kinetics of cathepsin D maturation in HeLa cells.** *Panel A*, HeLa cells were treaded for 4 h with 100 nM activated VacA (● in *B*) or with no toxin (■ in *B*), and metabolically labeled with ^{35}S for 30 min. At indicated chasing times, cathepsin D from dissolved cells (*C*) and extracellular media (*M*) were immunoprecipitated, run on SDS-PAGE, and subjected to autoradiography, as described under "Materials and Methods." Procathepsin D (53 kDa) and intermediate (47 kDa) and mature cathepsin D (31 kDa) are indicated by their molecular mass on the *left. Panel B*, quantification of autoradiography of the representative experiment reported in *panel A*.

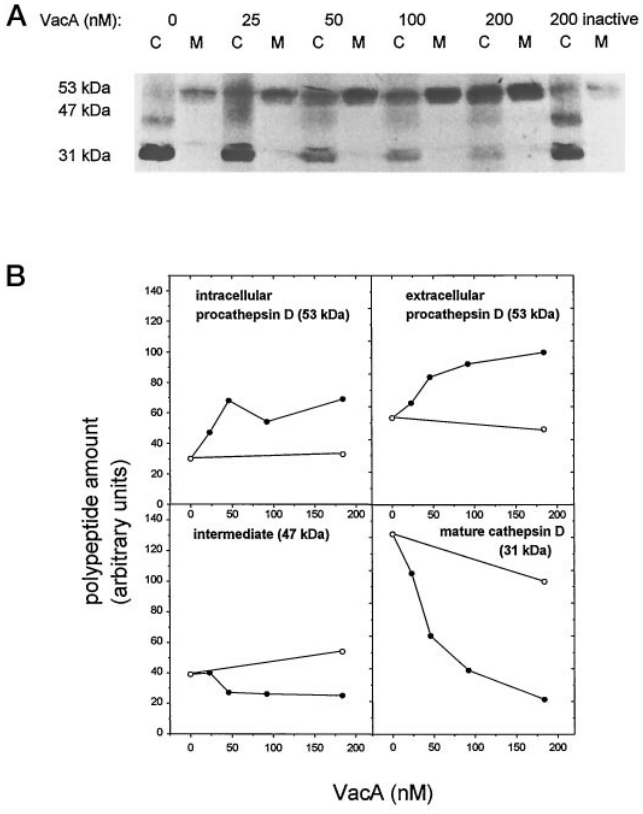

FIG. 2. **Dose response of VacA action on cathepsin D maturation in HeLa cells.** *Panel A*, cells were intoxicated for 4 h with indicated concentrations of either activated (● in *B*) or inactive (○ in *B*) VacA, pulsed with ^{35}S, and further chased for 2 h. Cathepsin D was then isolated by immunoprecipitation from cellular lysates (*C*) and extracellular media (*M*), run on SDS-PAGE, and subjected to autoradiography. Doses of VacA are shown at the *top*, while procathepsin D (53 kDa) and intermediate (47 kDa) and mature cathepsin D (31 KDa), indiated by molecular mass, are shown on the *left. Panel B*, quantification of the representative experiment shown in *panel A*.

RESULTS

VacA Alters Targeting and Intracellular Maturation of Procathepsin—To test the effect of VacA on procathepsin D maturation, HeLa cells were incubated with purified toxin, activated by preincubation at pH 2.0 as described previously (19), unless otherwise indicated. After a few hours, cells were washed, sulfur-starved, pulsed for 30 min with ^{35}S, and chased for different time periods in the presence of 5 mM M6P. Metabolically labeled cathepsin D was immunoprecipitated from cell lysates and extracellular media with specific rabbit polyclonal antibodies. The relative amounts of procathepsin D (53 kDa) and intermediate (47 kDa) and mature (31 kDa) forms of cathepsin D were determined by 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% (Fig. 1). It is worth noting that the reduced formation of mature cathepsin D is associated with an increased extracellular secretion of procathepsin D. Dose-response analysis, reported in Fig. 2, indicates that extracellular secretion and intracellular accumulation of procathepsin D account almost equally for the decrease of cathepsin D maturation. In addition, it is shown that VacA acts at relatively low doses with an IC_{50} of about 40 nM, and that preactivation by low pH is required for optimal inhibition of cathepsin D maturation.

VacA Inhibits Intracellular Degradation of EGF—EGF is a well characterized indicator of the endocytic pathway from the plasma membrane to lysosomes (43). After exposure to 200 nM 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 control and VacA-treated cells (not shown). Fig. 3 (*panel A*) shows that the kinetics of ^{125}I-EGF degradation was largely decreased upon VacA treatment and that this was accompanied by the intracellular accumulation of nondegraded EGF, since the extent of recycling of intact EGF into the extracellular medium was undiminished (Fig. 3, *panel B*). This latter fact is consistent with an effect of VacA on a step along the endocytic pathway after the early endosomes stage. Inhibition of EGF degradation and of procathepsin D maturation by VacA takes place in the same range of toxin concentrations, and both effects require activation of VacA by low pH treatment (Fig. 3, *panel C*). VacA does not affect the rate of endocytosis and recycling of ^{125}I-Tfn, which enters cells through a clathrin-dependent mechanism and is recycled to the plasma membrane, after discharge of bound iron in early endosomal compartments (48). As illustrated in Fig. 4, neither the initial rate of ^{125}I-Tfn endocytosis nor its ensuing release into the extracellular medium was significantly inhibited by VacA.

VacA Does Not Interfere with Intoxication of HeLa Cells by Diphtheria Toxin and Ricin—The specificity of VacA action on procathepsin D maturation and EGF degradation was further tested by using DT and ricin as markers of intracellular up-

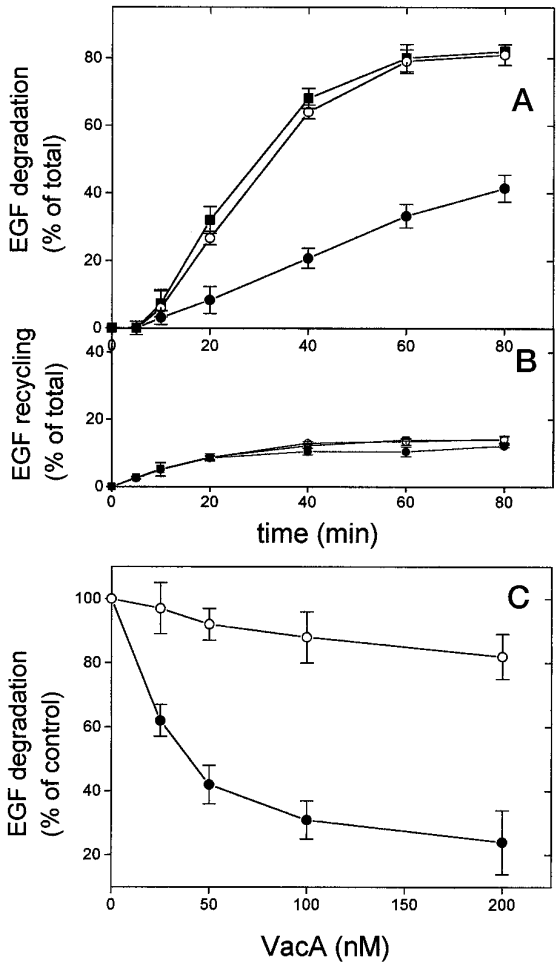

FIG. 3. **Effect of VacA on degradation and recycling of EGF by HeLa cells.** Cells were treated with activated (●), inactive (○) 200 nM VacA, or no toxin (■) for 4 h, incubated with ^{125}I-EGF (50 ng/ml) for 10 min, washed, and further incubated in DMEM as described under "Materials and Methods." At the indicated time, trichloroacetic acid-soluble (*panel A*) or -insoluble (*panel B*) radioactivity was determined in the extracellular medium and expressed as percent of total. In the experiments represented in *panel C*, cells were intoxicated as above with increasing concentrations of activated (●) or inactive (○) VacA, and the rates of EGF degradation were determined as in *panel A*. Data are expressed as percent with respect to control cells (treated with no toxin). Data are the means of four experiments run in duplicate, and *bars* represents ±S.E.

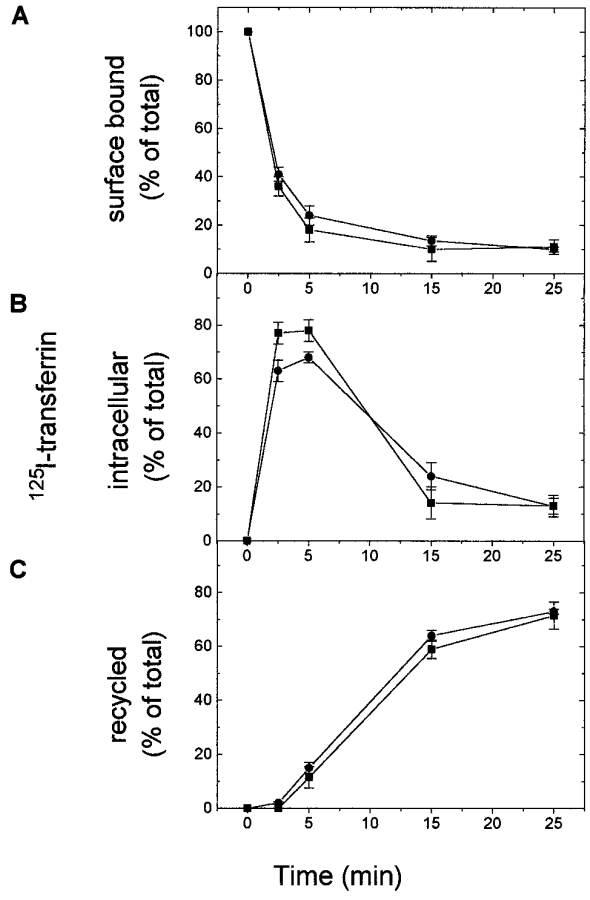

FIG. 4. **Endocytosis and recycling of transferrin in HeLa cells intoxicated with VacA.** Cells, treated as described in the legend of Fig. 3 with (●) or without toxin (■), were incubated for 1 h at 4 °C in the presence of 50 ng/ml ^{125}I-Tfn, washed, and further incubated in DMEM at 37 °C. At indicated times, membrane-bound (*A*), intracellular (*B*), and extracellular (*C*) Tfn were measured as described under "Materials and Methods." Data are the mean of three independent experiments run in duplicate, and *bars* represent ±S.E.

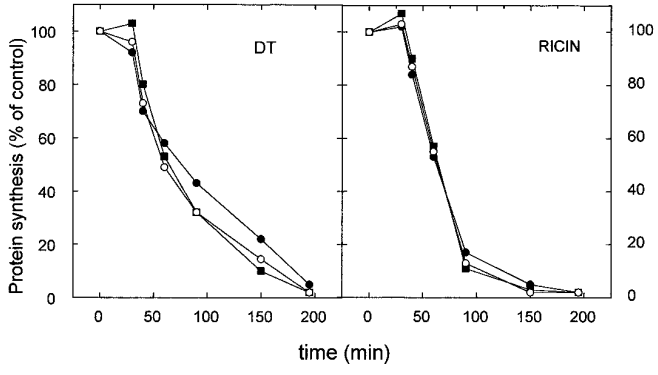

FIG. 5. **Kinetics of diphtheria toxin and ricin intoxication of HeLa cells treated with VacA.** Cells pretreated for 4 h with inactive VacA (○), low pH activated VacA (●), or with no toxin (■), were incubated with diphtheria toxin (*left panel*) or ricin (*right panel*). Incorporation of [^{14}C]leucine was determined at indicated times over a period of 4 min and referred to control uptake rate (cells with no DT or ricin). Data are the means of two independent experiments run in duplicate. S.E., here omitted for clarity, never exceeded 15%.

take. Such experiments are based on the fact that DT and ricin follow specific and distinct pathways inside eukariotic cells and can therefore be used as tools to probe alterations in membrane traffic. After endocytosis via a clathrin-dependent mechanism, in the case of DT or a clathrin-independent mechanism, in the case of ricin (53), and transport to early endosomes, the intracellular routes of the two toxins diverge. While DT delivers its enzymatic domain into the cytosol from an early endocytic compartment (49, 54), ricin needs to be transported retrogradely to TGN and from there to endoplasmic reticulum to intoxicate cells (55–57). Their intoxication kinetics can be easily followed by measuring the rate of protein synthesis, which depends strictly on the delivery of their catalytic subunit into the cytosol (58). As reported in Fig. 5, neither the lag phase, a parameter related to the time necessary for a toxin to reach the intracellular compartments where translocation takes place, nor the half-time of inhibition of protein synthesis by neither DT nor ricin was significantly affected by VacA.

Procathepsin D and Nondegraded EGF Accumulate in Lysosomes of Cells Intoxicated with VacA—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 implies their lysosomal accumulation. To discriminate between

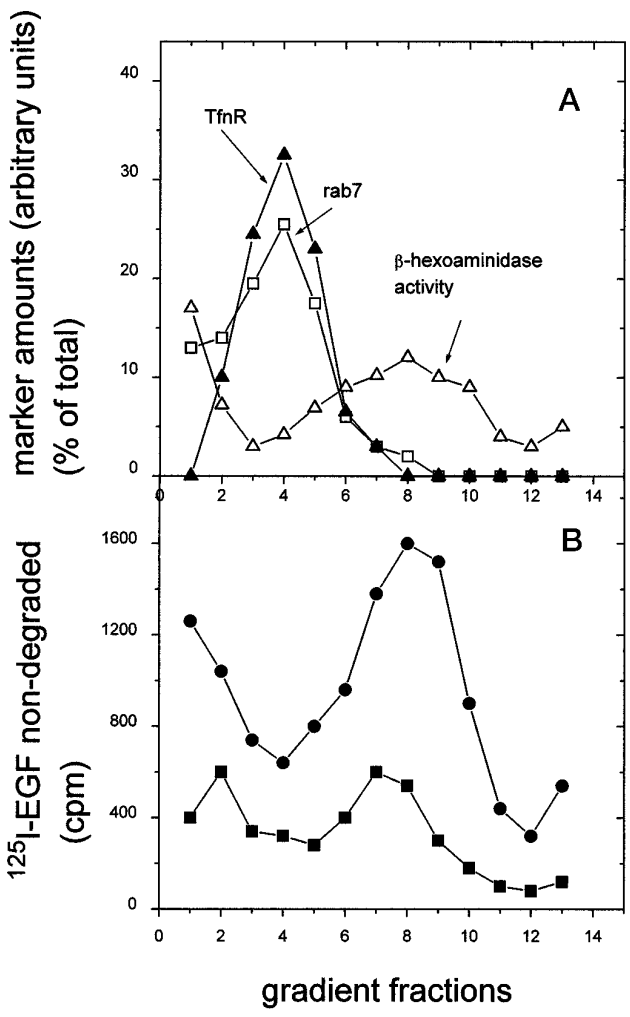

FIG. 6. **Intracellular localization of nondegraded ^{125}I-EGF in HeLa cells intoxicated with VacA.** Cells were treated with 200 nM activated VacA (●) or with no toxin (■) for 4 h, homogenized, and fractionated by ultracentrifugation on a 2 to 22% (w/v) Ficoll velocity gradient (see "Materials and Methods"). One-ml fractions were collected from the top of tubes, and the amount of specific markers of lysosomes (β-hexoaminidase, △), early endosomes (transferrin receptor, ▲), and late endosomes (rab7, □) was determined (*panel A*). The distribution of trichloroacetic acid-insoluble ^{125}I-EGF is shown in *panel B*.

these two possibilities, homogenates from control or VacA-treated cells were fractionated by ultracentrifugation on a Ficoll linear gradient (2 to 22%, w/v), adapted from Cutler and Cramer (59). This gradient system allows the separation of slowly sedimenting organelles, such as early and late endosomes and Golgi membranes, from lysosomes. The activity of the acidic hydrolase β-*N*-acetylglucosaminidase was used to detect lysosomes, while transferrin receptor and the small GTP-binding protein rab7 were used as markers of early and late endosomes, respectively (29, 60). In intoxicated cells nondegraded ^{125}I-EGF colocalized with lysosomal fractions rather than with endosomes (Fig. 6). This suggests that in VacA-affected cells internalized EGF molecules can reach lysosomes, but are not digested efficiently there. A VacA-induced deficiency of the intrinsic proteolytic activity at the late stages of the endocytic pathway is also suggested by the intracellular distribution of procathepsin D, shown in Fig. 7. VacA-treated and control cells were pulse-labeled with ^{35}S for 30 min or pulsed and chased for 45 or 120 min. Corresponding cell homogenates were fractionated on a 2 to 22% (w/v) Ficoll velocity gradient, and the various forms of cathepsin D were immunoprecipitated from a pool of light membrane fractions (fractions 2–5 of Fig. 7) and a pool of denser organelles (fractions 6–11 of Fig. 7). After 30 min of metabolic labeling, procathepsin D is present exclusively in the upper fractions of the gradient, where endoplasmic reticulum and Golgi membranes are collected and separated from lysosomes by the gradient system employed here. 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 non- 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. On the contrary, after a 45-min chase the amount of procathepsin D in the Golgi-endosomes fractions is much lower, due to release in the extracellular medium, and the amount of total enzyme that reaches lysosomes does not vary significantly, after correction for intracellular retention of procathepsin D. These data prove that procathepsin D and EGF are sorted to lysosomes in VacA-treated cells but that proper proteolysis does not occur.

VacA Partially Neutralizes Endosomal/Lysosomal pH—Cathepsin D processing, EGF degradation, and intracellular retention of procathepsin D require an acidic lumenal pH in the terminal part of the endocytic pathway (late endosomes and lysosomes). Not only low pH activates acidic proteases responsible for procathepsin D partial cleavage and for the complete degradation of EGF (34, 61, 62), but it also causes dissociation of hydrolases from M6P receptors inside late endosomes (31, 33). 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, washed, and chased for 15 min to 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. The mean pH value of endosomal/lysosomal compartments of several cells was then measured (50). Fig. 8 shows that only VacA activated by low pH treatment can raise the endosomal/lysosomal pH from the control value of about 5.25 to around 5.65. Such a variation, although apparently small, is in fact only slightly lower than that induced by 5 mM NH_4Cl, which brings the endosomal/lysosomal pH to 5.8. Moreover, the increased pH value caused by VacA is also significant when compared with the maximal neutralization obtained with 20 μM monensin (pH 6.2). The effects of VacA and 5 mM NH_4Cl on EGF degradation, procathepsin D secretion and processing, and pH increase inside acidic compartments are compared in the same figure. Such comparison indicates that 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.

DISCUSSION

Converging observations point to VacA as one of the major pathogenic factors in the development of gastritis and ulcers in men, subsequently to *H. pylori* infection (11). Despite its relevance and potential interest as a component of an anti-ulcer vaccine (9), the molecular mechanism of cell intoxication by VacA, as well as the functional implications of its activity *in vivo*, are largely undetermined. Here, we show that VacA modifies the intracellular sorting and processing of endogenous (procathepsin D) or exogenous (EGF) ligands directed toward lysosomes. Newly synthesized procathepsin D is secreted into the extracellular medium at a higher rate in intoxicated cells, indicating that segregation of acidic hydrolases from the default pathway of constitutive secretion, which operates at TGN, and the ensuing transport to endosomes is hampered by VacA. On the other hand, some newly synthesized procathepsin D,

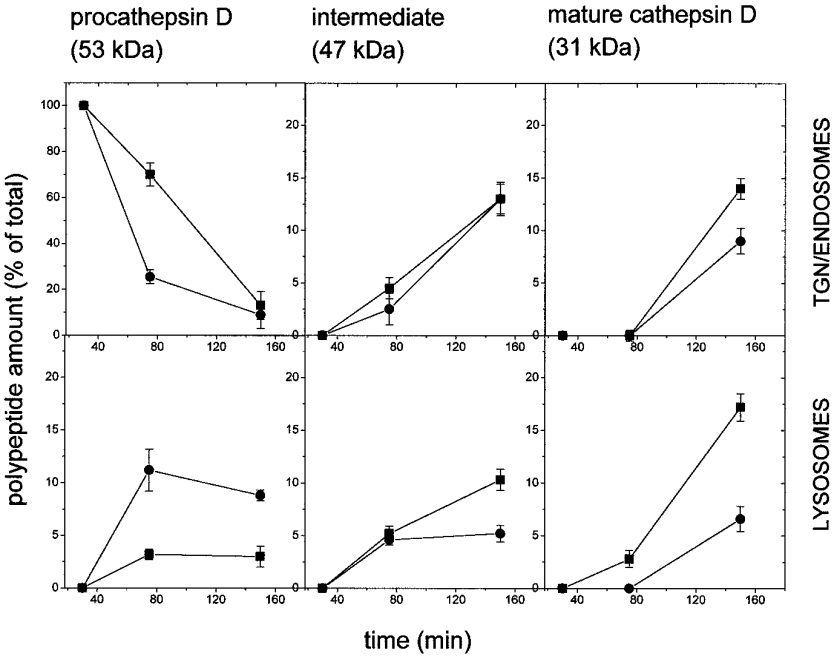

FIG. 7. **Intracellular distribution of different maturation forms of cathepsin D in VacA intoxicated HeLa cells.** Cells were intoxicated (●) or not (■) with activated VacA, labeled with ^{35}S, chased for the indicated times, and fractionated on a 2 to 22% Ficoll gradient as in the experiments described in Fig. 6. Indicated various forms of cathepsin D were then immunoprecipitated from pooled fractions 2–5 (corresponding to endosomes and TGN) and 6–11 (corresponding to lysosomes), run on SDS-PAGE, and quantitated after autoradiography. Data are the means of two independent experiments, and *bars* are ranges.

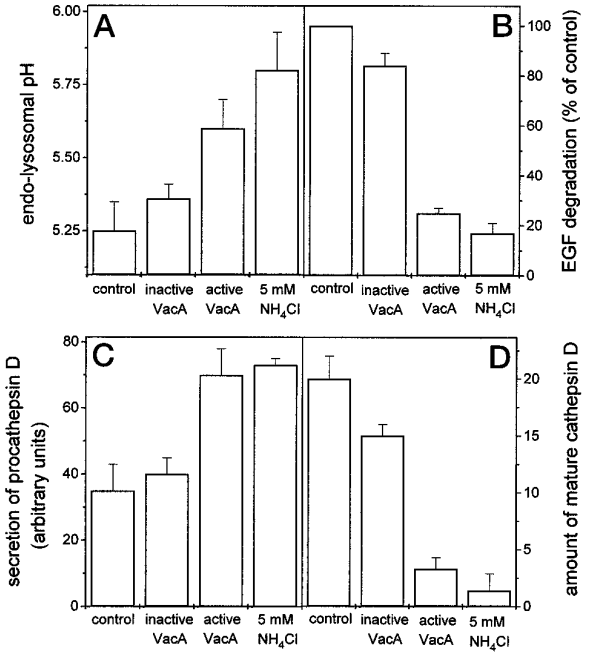

FIG. 8. **Effect of VacA and NH_4Cl on endosomal/lysosomal pH, EGF degradation, and procathepsin D maturation in HeLa cells.** Cells grown on glass coverslips were treated with inactive VacA, VacA-activated by exposure at pH 2.0 for 4 h, or with 5 mM NH_4Cl for 5 min as indicated. In same cases, cells were further incubated with FITC-dextran for 1 h and chased for 15 min, and the mean pH of the endocytic pathway was determined as described under "Materials and Methods" (*A*). Data represent the mean value of at least 100 cells for each condition, obtained in two independent experiments, and *bars* represent ranges. In other cases the rate of ^{125}I-EGF degradation (*B*) or the amount of mature cathepsin D inside cells (*D*) and of procathepsin D in the extracellular media (*C*) was measured as described in previous experiments. Values are the means of two experiments, and *bars* represent ranges.

instead of being secreted outside the cell, accumulates in lysosomes without being efficiently converted into the mature 31-kDa form. Degradation of endocytosed EGF is also depressed in VacA-treated cells, and EGF molecules are delivered to lysosomes, where their proteolysis is inhibited. At the same time transferrin recycling and the entry of diphtheria toxin and of ricin are unaltered in these cells.

Such effects can be attributed to a partial neutralization of the lumenal acidic pH of endosomal and lysosomal compartments in VacA-treated cells. In fact the activity of endosomal/lysosomal proteinases is lowered by pH neutralization, and hydrolases do not dissociate from the M6P receptor. The ability of VacA to partially neutralize the pH of the endosomal/lysosomal lumen appears to be a specific action of VacA rather than the secondary result of a general cell suffering. In fact, the use of highly purified toxin and omission of weak bases during intoxication allow obtaining such effects in the absence of macroscopic vacuolar degeneration. This is in agreement with the fact that many other energy-demanding cellular functions, such as protein synthesis, receptor-mediated endocytosis and recycling, constitutive secretion, transport to lysosome, and retrograde transport from endosomes to endoplasmic reticulum are not affected. In addition, the lack of protection from DT intoxication confirms that partial neutralization does not involve early endosomes but late endosomes and lysosomes. It is tempting to speculate that endocytosed VacA may alter the permeability of the endosomal and, eventually, lysosomal membrane, to protons, thus dissipating in part the lumenal acidity of these compartments. This hypothesis is in keeping with the recent detection of VacA within endocytic vesicles of intoxicated cells (63) and with the ability of recombinant COOH-terminal domain (p58) to increase monovalent cation leakage from liposomes (16).

The correlation between vacuolating activity and inhibition of lysosomal degradation, although not clarified, may have a practical application, providing a new quantitative and relatively easy assay, particularly in the case of measurement of ^{125}I-EGF degradation, to detect the presence and the relative activity of different VacA preparations or VacA toxoids.

The present findings may also bear on the pathogenesis of gastritis and ulcers. In fact, it is possible that a deficiency of intracellular digestion generates a condition similar to starvation in gastric epithelial cells, worsened by the concomitant deficiency in the lysosomal-targeting hydrolases, which would contribute to cell damage induced by other bacterial products or by factors released during chronic inflammation. More intriguing is the possibility that increased release of hydrolases into the extracellular medium, otherwise destined to lyso-

somes, may disrupt in part the extracellular matrix, the mucus layer covering the gastric cells, or the adhesion and junction complexes between epithelial cells. This may result in an increased leak of nutrients through the gastric epithelium, favoring *H. pylori* survival and growth.

In conclusion, two new specific actions of VacA on cells have been uncovered: (i) induction of increased extracellular secretion of acidic hydrolases and (ii) impairment of the degradative power of late endosomes and lysosomes. Mistargeting of acidic hydrolases as well as their decreased proteolytic activity inside the cell can be accounted for by the ability of VacA to partially neutralize the acidic pH of the lumen of endosomes and lysosomes.

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Effect of *Helicobacter pylori* Vacuolating Toxin on Maturation and Extracellular Release of Procathepsin D and on Epidermal Growth Factor Degradation

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