PIKfyve Kinase and SKD1 AAA ATPase Define Distinct Endocytic Compartments

ONLY PIKfyve EXPRESSION INHIBITS THE CELL-VACUOLATING ACTIVITY OF HELICOBACTER PYLORI VacA TOXIN

Ognian C. Ikonomov‡, Diego Sbrisșa‡, Tamotsu Yoshimori§, Timothy L. Cover¶, and Assia Shisheva‡‡

Received for publication, June 21, 2002, and in revised form, August 7, 2002 Published, JBC Papers in Press, September 3, 2002, DOI 10.1074/jbc.M208068200

The mammalian phosphatidylinositol (PtdIns)-5-P/PtdIns-3,5-P2-producing kinase PIKfyve and AAA ATPase SKD1, as their yeast counterparts, are implicated in the formation and function of multivesicular bodies/late endosomes. Point mutations inhibiting the enzyme activities convert PIKfyve and SKD1 into dominant-negative mutants (PIKfyveK1831E and SKD1E235Q), whose expression in cells of kidney origin induces a vacuolation phenotype. This phenotype closely resembles the changes in late endosomal-lysosomal morphology that occur following cell exposure to the vacuolating cytotoxin (VacA) from Helicobacter pylori. Here we have examined the possible functional relationship between PIKfyve and SKD1 as well as the role of these enzymes in the molecular mechanism of VacA-induced intracellular vacuolation. When co-expressed in COS cells, PIKfyveWT reduced SKD1E235Q-dependent vacuole formation, whereas SKD1WT did not alter the vacuolation induced by PIKfyveK1831E. In addition, SKD1E235Q disrupted the normal distribution of PIKfyveWT. Expression of PIKfyveWT in COS and HEK293 cells inhibited vacuolation induced by subsequent intoxication with VacA, and microinjection of the PIKfyve lipid product PtdIns-3,5-P2 produced a similar inhibitory effect. In contrast, in COS cells expressing SKD1WT, VacA induced the formation of characteristic vacuoles with an efficiency similar to that in the control cells. These observations demonstrate that, although PIKfyve and SKD1 are functionally related, only PIKfyve regulates VacA action, and suggest that the inhibition of PIKfyve PtdIns-3,5-P2-producing activity is a key molecular event in VacA-induced cellular vacuolation.

Despite considerable clinical and basic research efforts, an understanding of the molecular mechanisms whereby the Gram-negative bacterium Helicobacter pylori induces gastritis and peptic ulcers in humans is still elusive. Most H. pylori strains secrete a cytotoxin, VacA, which causes extensive vacuolation in epithelial cells (for a recent review, see Ref. 1). VacA-induced vacuoles represent an aberrant intracellular hybrid compartment that forms as a result of a heterotypic fusion between late endosomes and lysosomes (2, 3). Little is known about the intracellular mechanisms that control VacA-induced reorganization of the late endosomal compartment. A role for several proteins involved in membrane trafficking, such as Rab7, Rab1, and dynamin, has recently been proposed (4–6). However, since none of these proteins nor their loss- or gain-of-function mutants mimic the VacA phenotype (4–6), other proteins and mechanisms relevant to late endosomal compartment biogenesis and function are likely to be involved.

While the broad outline of the membrane traffic through the endocytic pathway from the plasma membrane to the late endosome fusion with the lysosomes (or vacuoles in yeast) is now well established, the molecular mechanism(s) underlying the formation and sorting functions of the late endosomal compartment are poorly understood (for recent reviews, see Refs. 7 and 8). Morphologically, late endosomes appear to contain multiple small vesicles, which originate by invagination and pinching off from the limiting membrane into the lumenal space. Because of this multivesicular appearance, late endosomes have also been referred to as multivesicular bodies (MVBs); both terms are used herein interchangeably. Genetic studies in budding yeast have identified a set of genes, the class E genes, that are essential in MVB formation (9). Electron microscopy studies of class E vps mutants have documented the presence of an enlarged, aberrant endosomal compartment comprised of stacked membranes (9). A central role among the class E proteins is assigned to Vps4p, an AAA ATPase, whose on/off membrane cycle coupled with ATP hydrolysis regulates the membrane association of other class E proteins (10). A regulatory role in MVB formation is also attributed to Fab1p lipid kinase and its product PtdIns-3,5-P2. Yeast cells that lack Fab1p function contain abnormally large vacuoles and, like the class E mutant strains, appear to be defective in the formation of MVBs (7, 9). Thus, genetic studies in yeast strongly suggest that the ability to properly invaginate endosomal membranes and to form MVBs is largely dependent on the correct function of Fab1 and Vps4 gene products.
nant-negative mutants. Similar to the morphological changes observed in yeast, expression of ATPase-deficient SKD1 or kinase-deficient PIKfyve in mammalian cells of kidney origin induces peripheral vacuoles and enlarged vesicles (13–16). This phenotype is highly reminiscent of the cellular alterations induced by H. pylori VacA. Expressed SKD1 or PIKfyve typically localize on the membranes that outline dilated endomembrane structures. SKD1 vesicles appear positive for markers of either the early or late endocytic compartments, whereas PIKfyve predominantly co-localizes with MVB markers and not with markers for the earlier compartments (13–17). These observations suggest that the two mutants dilate both distinct and overlapping endocytic structures. The dramatic vacuolation induced by PIKfyve is corrected by high levels of PIKfyve or PtdIns-3,5-P2, implying that PIKfyve-originated production of PtdIns-3,5-P2 maintains mammalian cell morphology and endocytic membrane homeostasis (15, 16). The overall evolutionary conservation of the molecular events involved in endocytosis together with the conservation of SKD1 and PIKfyve cellular roles in yeast and mammals suggest that, like their yeast orthologs, mammalian SKD1 and PIKfyve may be functionally related. In this context we sought to examine, first, the functional relationship between the endocytic compartments defined by PIKfyve and SKD1 in mammalian cells and, second, to investigate the potential role of these enzymes in VacA-induced vacuolation. Our results suggest a functional connection between the two proteins, and indicate that PIKfyve, through PtdIns-3,5-P2 production, but not SKD1, negatively regulates the VacA-induced endomembrane vacuolation.

EXPERIMENTAL PROCEDURES

DNA Constructs and Transient Cell Transfection—Generation and characterization of pEGFP-SKD1 and pEGFP-SKD1 were previously published (16) or pCMV5-HA-PIKfyve, pEGFP-HA-PIKfyve, and pCMV5-HA-PIKfyve were described previously (17–19). COS-7 cells, maintained as described previously (15), were seeded on 22 × 22-mm coverslips (35-mm dishes) and transfected with the cDNA constructs indicated in the figure legends using LipofectAMINE (Invitrogen) as a transfection reagent. Twenty-four hour posttransfection the cells were processed for fluorescence microscopy or further treated with activated VacA toxin.

Generation of Stable Cell Lines—A stably transfected doxycycline-induced (Tet-On) cell line inducibly expressing PIKfyve was generated following the Tet-Off/Tet-On gene expression systems manual (Clontech). Briefly, PIKfyve cDNA (released by XbaI-SalI from pBluescript IISK (19) together with an HA-encoding adapter (flanked with BamHI and XbaI restriction sites) were cloned into the BamHI-SalI site of the pTRE2hyg vector. The expected organization of the construct was confirmed by restriction mapping. The pTRE2hygHA-PIKfyve vector, linearized by SalI, was used to transfect a parental HEK293 Tet-On cell line (Clontech) by LipofectAMINE as a transfection reagent. Transfected cells were selected by hygromycin treatment at 125 μg/ml, a concentration found to eliminate all susceptible cells after 5–7 days of treatment. Individual cell clonal lines were isolated by cloning cylinders, propagated, and then probed for a doxycycline-inducible recombinant PIKfyve by Western blotting with anti-HA polyclonal antibodies (a gift of Mike Czech) and immunofluorescence microscopy with anti-HA monoclonal antibody (a gift of Steve Doyle).

VacA Toxin Purification, Activation, and Cell Treatment—VacA purified from H. pylori 60190 culture supernatant was activated before use by a dose-wise addition of 0.2 M HCl to pH 3 as described elsewhere (20). Cells were treated with the activated toxin (10 μg/ml final concentration) in the growing media supplemented with 20 μM HEPES, pH 7.4, and 5 mM NH4Cl for a time interval indicated in the figure legends.

Fluorescence Microscopy—HA-PIKfyve proteins were detected by indirect immunofluorescence microscopy using an anti-HA monoclonal antibody and Texas Red-conjugated goat anti-mouse secondary antibody. Cell fixation and washing conditions were described previously (17). EGFP-based proteins were detected by GFP signals. Fluorescence analyses were performed with a digital imaging fluorescent microscope (Nikon Eclipse TE200) using a 40× Hoffman Modulation Contrast objective or Nikon Apo DM 60/1.4 immersion lens as indicated in the figure legends. Visible vacuoles were assessed by the phase-contrast and the above objectives. Representative images were captured by a SPOT RT Slider charge coupled device camera (Diagnostic Instruments) and processed further by SPOT 3.2 software.

Cell Microinjection—COS-7 cells, grown on coverslips, were transferred to a Leibowitz-15 medium and microinjected in the cytoplasm with a semiautomatic microneedle (Eppendorf micromanipulator 5171 and Femtojet 5247) as described previously (16). Briefly, cells were injected with the indicated PIs mixed with Texas Red-dextran (70,000, Molecular Probes) or with Texas Red-dextran alone and then returned to a complete medium to recover for 2 h at 37 °C. Cells were then treated overnight with VacA toxin and observed by fluorescence microscopy following the above objectives. The presence or absence of visible vacuoles were assessed by phase-contrast microscopy. For data quantitation, the vacuolating effects of VacA in injected (dextran-positive) or non-injected cells within the same dish were calculated as percentage of the total number of counted cells (>100 cells/condition) in three independent experiments and are presented as mean ± S.E.

PIKfyve Lipid Kinase Activity—To examine whether VacA had a direct effect on PIKfyve lipid kinase activity in vitro, PIKfyve immunoprecipitates were preincubated for 15 min at 37 °C with activated VacA (0.3–10 μg/ml final concentration), followed by subsequent analysis of the generated radiolabeled products, as described previously (18, 21).

SDS PAGE and Immunoblotting—Cell lysates were subjected to SDS-PAGE (6% gel), and after electrophoresis, the membrane was probed with anti-HA polyclonal antibodies as described previously (15, 21).

Quantitation of VacA-induced Vacuolation by Neutral Red—Twenty-four hours following induction of protein expression in HEK293 stable (PIKfyve wt, clone 9) or parental clones in the presence or absence of doxycycline, cells seeded on 12-well plates were left untreated or treated with VacA for 5 h (triplicates/condition). The cells were then allowed to take up neutral red for 4 min as described previously (22). Following washings, neutral red was extracted from cells with acidified alcohol. The absorbance (540 nm) of the samples was measured with a spectrophotometer (Beckman DU-50). The net neutral red accumulation was calculated by subtracting the absorbance of non-treated cells from the values of the VacA-treated samples.

RESULTS AND DISCUSSION

We have previously observed that expression of enzymatically defective, dominant-negative mutants of PIKfyve and SKD1 in cells of kidney origin induce similar endomembrane vacuolation. The mutant proteins were found to populate related endocytic compartments as defined by the mannose 6-phosphate receptor marker (14–16). Fig. 1 illustrates typical images of the abnormal phenotypes in COS-7 cells transiently expressing SKD1 or PIKfyve, where multiple enlarged vesicles and endomembrane vacuoles could be readily observed. To examine a possible functional relationship between these two proteins we co-expressed SKD1 and PIKfyve wild type proteins and/or their dominant-negative mutants in COS-7 cells. Co-expression of SKD1 WT and PIKfyve WT did not change the typical localization pattern seen previously with the singly expressed proteins (14–17). Thus, pEGFP-SKD1 WT showed the characteristic diffuse cytosolic and nuclear localization and co-expressed PIKfyve WT displayed the typical extranuclear scattered puncta on a background of a diffuse cytosolic signal (Fig. 2, a and b). No noticeable morphological alterations were found in cells co-expressing the wild types (Fig. 2c). However, co-expression of ATPase-deficient SKD1 and PIKfyve WT resulted in several changes in protein localization as well as in cell morphology. Thus, in the presence of PIKfyve WT, the SKD1-positive structures appeared as fine perinuclear puncta (Fig. 2d). This differed drastically from the SKD1-positive dilated vesicles and vacuoles observed previously by the singly expressed SKD1 in cells of kidney origin and confirmed here (Refs. 13 and 14 and Fig. 1, a and b). The PIKfyve WT-positive compartment also underwent a significant redistribution in the presence of co-expressed SKD1 and was seen as perinuclear clusters,
rather than scattered puncta (Fig. 2e). In contrast to this PIKfyveWT-dependent inhibition of the SKD1E235Q-induced abnormal morphology, SKD1WT did not prevent the abnormal phenotype produced by dominant-negative PIKfyveK1831E. As demonstrated in Fig. 2, g–i, cells co-expressing SKDWT and PIKfyveK1831E produced a vacuolation similar to that observed upon expression of PIKfyveK1831E alone. Finally, the vacuolation in cells co-expressing the two dominant-negative mutants appeared as a sum of their individual abnormal phenotypes with only partial co-localization of the two proteins upon merging the images (Fig. 2, j–l, and not shown). These observations are consistent with the notion that expressed SKD1E235Q and PIKfyveK1831E dilate both overlapping and distinct populations of endocytic structures, which are functionally related. The fact that increased PIKfyve enzymatic activity could largely overtake the action of the ATPase-deficient SKD1 mutant, but not vice versa, indicates that the functions of the two proteins are not interchangeable. Rather, we speculate the two enzymes act vice versa, indicates that the functions of the two proteins are not interchangeable. Rather, we speculate the two enzymes act

vacuolation caused by the H. pylori VacA toxin. Accumulated experimental evidence supports two hypotheses for the molecular mechanism of VacA action: the toxin forms anion-selective channels and/or interacts with protein targets that trigger the endogenous vacuolating mechanism (reviewed in Ref. 1). In this regard endosomal proteins, such as SKD1 and PIKfyve, whose dominant-interfering mutants mimic VacA vacuolation should be a central focus, since they are likely candidate downstream targets of the VacA toxin. Therefore, we examined the effect of SKD1WT and PIKfyveWT expression on VacA-induced vacuolation. For this purpose transfected COS-7 cells were treated for 24 h with VacA and the number of the SKD1WT- or PIKfyveWT-expressing cells that displayed a vacuolated phenotype, by phase-contrast of the same fields (Fig. 3). Arrows in d–f demonstrate lack of a vacuolation phenotype in SKD1E235Q/PIKfyveWT co-expressing cell, and arrowheads indicate the presence of a vacuolation phenotype in a cell expressing only SKD1E235Q. Bar, 10 μm.
PIKfyve and SKD1 in VacA-induced Vacuolation

The dramatic inhibition of VacA-induced vacuolation by two independent approaches, i.e. expression of PIKfyve WT in different cell types and cytosolic microinjection of PtdIns-3,5-P2, demonstrated above, strongly supports the central role of PIKfyve and its lipid product PtdIns-3,5-P2 in the molecular mechanisms of endomembrane vacuolation induced by VacA. However, VacA does not directly inhibit PIKfyve, because, when added to the in vitro PIKfyve kinase assay, the toxin did not inhibit PtdIns-3,5-P2 production (not shown). This result is consistent with the notion that VacA exerts an indirect inhibitory effect on this enzyme. Determination of the intracellular PtdIns-3,5-P2 levels in response to VacA as well as the pre-

- **Fig. 3. Expression of PIKfyve WT, but not SKD1 WT, prevents H. pylori VacA-induced vacuolation.** COS-7 cells were transfected with the indicated pEGFP-based constructs and intoxicated with VacA as specified under “Experimental Procedures.” The cells were fixed 24 h after VacA addition and observed by fluorescence (a and c) and phase-contrast microscopy of the same fields (b and d). A, shown are cells expressing SKD1 WT (panel a) that display large perinuclear vacuoles similar to those of the non-transfected cells (panel b), a PIKfyve WT, expressing cell (panel c) that failed to be vacuolated by VacA, in contrast to the neighboring cells (panel d). Bar, 10 μm. B, quantitation of vacuolating effects of VacA from four independent experiments assessed by appearance of visible vacuoles through phase-contrast microscopy. The number of vacuole-positive cells is expressed as percentage of the total number of counted cells (>100 cells/condition/experiments) and presented as mean ± S.E.

- **Table:**

| Transfections | PIKfyve WT | SKD1 WT |
|---------------|------------|---------|
|                | -          | -       |
|                | +          | -       |
|                | -          | +       |

induction of the protein expression with doxycycline as revealed by Western blotting analysis with anti-PIKfyve antibodies (Fig. 4A). The expressed protein seems, at least in part, to localize to the authentic PIKfyve sites as judged by its vesicular appearance, along with the cytosolic staining upon immunofluorescence microscopy analysis with an anti-HA antibody (Fig. 4B). Importantly, induction of the PIKfyve WT expression resulted in a substantial inhibition of the cellular vacuolation due to VacA as revealed on the basis of neutral red uptake of the vacuolated cells (Fig. 4C). Thus, 5 h following intoxication with VacA, HEK293 cells that were induced to express PIKfyve WT showed 60% less net neutral red uptake versus non-induced cells (Fig. 4C). Importantly, doxycycline treatment did not affect the basal or VacA-dependent neutral red uptake in the parental HEK293 cells (Fig. 4C). It should be noted that at the level of expression of PIKfyve WT in this cell type, 24 h following VacA treatment, both induced and non-induced cells were vacuolated and showed similar values of the neutral red uptake (not shown). However, expression of PIKfyve WT in this cell type not only substantially inhibited early VacA induced vacuolation but also led to a faster recovery of cell morphology back to normal after removal of VacA. As seen on the images presented in Fig. 4D, 18 h following the toxin removal, HEK293 cells induced to express PIKfyve WT showed practically no vacuoles. In contrast, the non-induced cells displayed prominent vacuolation (Fig. 4D). Together, these results demonstrate that while dominant-negative mutants of SKD1 and PIKfyve could both induce a vacuolated phenotype, only high levels of PIKfyve WT inhibit the vacuole formation due to VacA. These observations suggest that VacA-dependent vacuoles arise in a process that is negatively regulated by PIKfyve. SKD1 action is likely upstream or distal to the site of VacA action on endocytic membranes.

Having established that high levels of PIKfyve negatively regulate the VacA-induced endomembrane defects, we next examined whether this effect is mediated by the PIKfyve lipid product PtdIns-3,5-P2. This is particularly important, since PIKfyve produces several different products (PtdIns-3,5-P2, produces PtdIns-5-P and phosphoprotein(s); Refs. 16, 18, 21, and 23), each of which might contribute to the ability of PIKfyve WT to inhibit VacA-induced vacuole formation. Therefore, we next examined whether increased cellular levels of PtdIns-3,5-P2 could selectively inhibit the VacA-induced vacuolation in COS cells. As shown in Fig. 5, microinjection of PtdIns-3,5-P2, but not PtdIns-4,5-P2 or PtdIns-5-P (not shown), inhibited the capacity of VacA to induce cell vacuolation. Quantitation from three independent microinjection experiments indicates that only 15 ± 2% of the PtdIns-3,5-P2-injected cells exhibited vacuoles, notably with a less pronounced appearance compared with the PtdIns-4,5-P2-injected or non-injected cells, in which the VacA efficiency was 60 ± 5% (p < 0.001). Thus, similarly to the vacuolation induced by the kinase-defective dominant-negative PIKfyve mutants (16), the VacA-induced vacuolation was selectively rescued by increased PtdIns-3,5-P2 levels.

To confirm the observed inhibition of VacA-induced vacuolation by PIKfyve WT in another cell system and to provide alternative quantification of this phenomenon, we examined the vacuolating effect of VacA in a HEK293 stable line inducibly expressing PIKfyve WT. These cells express ~7- to 8-fold higher levels of PIKfyve WT versus the endogenous protein 24 h after induction of the protein expression with doxycycline as revealed by Western blotting analysis with anti-PIKfyve antibodies (Fig. 4A). The expressed protein seems, at least in part, to localize to the authentic PIKfyve sites as judged by its vesicular appearance, along with the cytosolic staining upon immunofluorescence microscopy analysis with an anti-HA antibody (Fig. 4B). Importantly, induction of the PIKfyve WT expression resulted in a substantial inhibition of the cellular vacuolation due to VacA as revealed on the basis of neutral red uptake of the vacuolated cells (Fig. 4C). Thus, 5 h following intoxication with VacA, HEK293 cells that were induced to express PIKfyve WT showed 60% less net neutral red uptake versus non-induced cells (Fig. 4C). Importantly, doxycycline treatment did not affect the basal or VacA-dependent neutral red uptake in the parental HEK293 cells (Fig. 4C). It should be noted that at the level of expression of PIKfyve WT in this cell type, 24 h following VacA treatment, both induced and non-induced cells were vacuolated and showed similar values of the neutral red uptake (not shown). However, expression of PIKfyve WT in this cell type not only substantially inhibited early VacA induced vacuolation but also led to a faster recovery of cell morphology back to normal after removal of VacA. As seen on the images presented in Fig. 4D, 18 h following the toxin removal, HEK293 cells induced to express PIKfyve WT showed practically no vacuoles. In contrast, the non-induced cells displayed prominent vacuolation (Fig. 4D). Together, these results demonstrate that while dominant-negative mutants of SKD1 and PIKfyve could both induce a vacuolated phenotype, only high levels of PIKfyve WT inhibit the vacuole formation due to VacA. These observations suggest that VacA-dependent vacuoles arise in a process that is negatively regulated by PIKfyve. SKD1 action is likely upstream or distal to the site of VacA action on endocytic membranes.

Having established that high levels of PIKfyve negatively regulate the VacA-induced endomembrane defects, we next examined whether this effect is mediated by the PIKfyve lipid product PtdIns-3,5-P2. This is particularly important, since PIKfyve produces several different products (PtdIns-3,5-P2, produces PtdIns-5-P and phosphoprotein(s); Refs. 16, 18, 21, and 23), each of which might contribute to the ability of PIKfyve WT to inhibit VacA-induced vacuole formation. Therefore, we next examined whether increased cellular levels of PtdIns-3,5-P2 could selectively inhibit the VacA-induced vacuolation in COS cells. As shown in Fig. 5, microinjection of PtdIns-3,5-P2, but not PtdIns-4,5-P2 or PtdIns-5-P (not shown), inhibited the capacity of VacA to induce cell vacuolation. Quantitation from three independent microinjection experiments indicates that only 15 ± 2% of the PtdIns-3,5-P2-injected cells exhibited vacuoles, notably with a less pronounced appearance compared with the PtdIns-4,5-P2-injected or non-injected cells, in which the VacA efficiency was 60 ± 5% (p < 0.001). Thus, similarly to the vacuolation induced by the kinase-defective dominant-negative PIKfyve mutants (16), the VacA-induced vacuolation was selectively rescued by increased PtdIns-3,5-P2 levels.

The dramatic inhibition of VacA-induced vacuolation by two independent approaches, i.e. expression of PIKfyve WT in different cell types and cytosolic microinjection of PtdIns-3,5-P2, demonstrated above, strongly supports the central role of PIKfyve and its lipid product PtdIns-3,5-P2 in the molecular mechanisms of endomembrane vacuolation induced by VacA. However, VacA does not directly inhibit PIKfyve, because, when added to the in vitro PIKfyve kinase assay, the toxin did not inhibit PtdIns-3,5-P2 production (not shown). This result is consistent with the notion that VacA exerts an indirect inhibitory effect on this enzyme. Determination of the intracellular PtdIns-3,5-P2 levels in response to VacA as well as the pre-

- **Fig. 3. Expression of PIKfyve WT, but not SKD1 WT, prevents H. pylori VacA-induced vacuolation.** COS-7 cells were transfected with the indicated pEGFP-based constructs and intoxicated with VacA as specified under “Experimental Procedures.” The cells were fixed 24 h after VacA addition and observed by fluorescence (a and c) and phase-contrast microscopy of the same fields (b and d). A, shown are cells expressing SKD1 WT (panel a) that display large perinuclear vacuoles similar to those of the non-transfected cells (panel b), a PIKfyve WT, expressing cell (panel c) that failed to be vacuolated by VacA, in contrast to the neighboring cells (panel d). Bar, 10 μm. B, quantitation of vacuolating effects of VacA from four independent experiments assessed by appearance of visible vacuoles through phase-contrast microscopy. The number of vacuole-positive cells is expressed as percentage of the total number of counted cells (>100 cells/condition/experiments) and presented as mean ± S.E.
dicted upstream intermediate of the PIKfyve pathway that is negatively regulated by VacA, are important objectives for future studies.

Thus far, three proteins, each localized on late endosomal-lysosomal membranes and displaying the ability to bind and hydrolize GTP, have been shown to be important in VacA-induced vacuole formation, i.e. Rab7 (4), Rac1 (5), and dynamin (6). Interestingly, these GTPases affect similarly the VacA action in that the expression of the dominant-negative mutants inhibits the formation of vacuoles, whereas expression of the wild type proteins or constitutively active mutants either has no effect or slightly augments the effect of the toxin (4–6).

These results are consistent with a hypothesis that VacA intoxication is associated with an intracellular increase of the active forms of Rac1, Rab7, or dynamin. The fact that none of the constitutively active mutants, by themselves, are able to mimic VacA suggests, however, additional mechanisms. In contrast to the action of GTPases, PIKfyve was able both to inhibit VacA-induced vacuolation upon increasing the cellular levels of its enzymatic activity or generated product and to mimic VacA vacuolation upon expression of enzymatically inactive dominant-negative mutants. These data suggest that VacA-dependent indirect inhibition of PIKfyve lipid kinase activity and reduced PtdIns-3,5-P2 production could potentially be the sole triggering mechanism involved in the toxin-induced vacuola-
tion. Whether and how Rac1, Rab7, and dynamin are involved in the endogenous vacuolation initiated by the PtdIns-3,5-P2 depletion remain to be identified.

In conclusion, we demonstrate here that unlike SKD1, PIKfyve, through its PtdIns-3,5-P2 producing activity, inhibits the vacuolation induced by the *H. pylori* VacA toxin. Among the candidate molecular intermediates, reported previously (4–6) and here, only dominant-negative PIKfyve could mimic VacA endomembrane vacuolation, thus making PIKfyve undoubtedly a very interesting VacA target for future clinical and fundamental studies.

REFERENCES
1. Papini, E., Zoratti, M., and Cover, T. L. (2001) *Toxicon* **39**, 1757–1767
2. Papini, E., de Bernard, M., Milia, E., Bugnoli, M., Zerial, M., Rappuoli, R., and Montecucco, C. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9720–9724
3. Molinari, M., Galli, C., Norais, N., Telford, J. L., Rappuoli, R., Luzio, J. P., and Montecucco, C. (1997) *J. Biol. Chem.* **272**, 25339–25344
4. Papini, E., Satin, B., Bucci, C., de Bernard, M., Telford, J. L., Manetti, R., Rappuoli, R., Zerial, M., and Montecucco, C. (1997) *EMBO J.* **16**, 15–24
5. Hotchin, N. A., Cover, T. L., and Akhtar, N. (2000) *J. Biol. Chem.* **275**, 14009–14012
6. Suzuki, J., Ohnshi, H., Shibata, H., Wada, A., Hirayama, T., Iri, T., Ueda, N., Kanamuru, C., Tsuchida, T., Mashima, H., Yasuda, H., and Fujita, T. (2001) *J. Clin. Invest.* **107**, 363–370
7. Odorizzi, G., Babst, M., and Emr, S. D. (2000) *Trends Biochem. Sci.* **25**, 229–235
8. Piper, R. C., and Luzio, J. P. (2001) *Traffic* **2**, 612–621
9. Odorizzi, G., Babst, M., and Emr, S. D. (1998) *Cell* **95**, 847–858
10. Babst, M., Wendland, B., Estepa, E. J., and Emr, S. D. (1998) *EMBO J.* **17**, 2982–2993
11. Scheuring, S., Boder, D., Rohricht, R. A., Muller, S., Beyer, A., and Kohrer, K. (1999) *Gene (Amst.)* **234**, 149–159
12. Shisheva, A. (2001) *Cell Biol. Int.* **25**, 1201–1206
13. Bishop, N., and Woodman, P. (2000) *Mol. Biol. Cell* **11**, 227–239
14. Yoshimori, T., Yamagata, F., Yamamoto, A., Mizushima, N., Kabeys, Y., Nara, A., Miwako, I., Ohashi, M., Ohsumi, M., and Ohsumi, Y. (2000) *Mol. Biol. Cell* **11**, 747–762
15. Ilkonov, O. C., Sbrissa, D., and Shisheva, A. (2001) *J. Biol. Chem.* **276**, 26141–26147
16. Ilkonov, O. C., Sbrissa, D., Milak, K., Kanzaki, M., Pessin, J., and Shisheva, A. (2002) *J. Biol. Chem.* **277**, 9206–9211
17. Shisheva, A., Rusin, B., Ilkonov, O. C., DeMarco, C., and Sbrissa, D. (2001) *J. Biol. Chem.* **276**, 11869–11869
18. Sbrissa, D., Ilkonov, O. C., and Shisheva, A. (2000) *Biochemistry* **39**, 10980–10989
19. Shisheva, A., Sbrissa, D., and Ilkonov, O. (1999) *Mol. Cell. Biol.* **19**, 623–634
20. Cover, T. L., Hanson, P. I., and Heuser, J. E. (1997) *J. Cell Biol.* **138**, 759–769
21. Sbrissa, D., Ilkonov, O. C., and Shisheva, A. (1999) *J. Biol. Chem.* **274**, 21586–21597
22. Cover, T. L., Puryear, W., Perez-Perez, G. I., and Blaser, M. J. (1991) *Infect. Immun.* **59**, 1264–1270
23. Sbrissa, D., Ilkonov, O. C., Deeb, R., and Shisheva, A. (2002) *J. Biol. Chem.* **277**, in press
PIKfyve Kinase and SKD1 AAA ATPase Define Distinct Endocytic Compartments: ONLY PIKfyve EXPRESSION INHIBITS THE CELL-VACUOLATING ACTIVITY OF HELICOBACTER PYLORI VacA TOXIN

Ognian C. Ikonomov, Diego Sbrissa, Tamotsu Yoshimori, Timothy L. Cover and Assia Shisheva

J. Biol. Chem. 2002, 277:46785-46790.
doi: 10.1074/jbc.M208068200 originally published online September 3, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M208068200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 22 references, 14 of which can be accessed free at http://www.jbc.org/content/277/48/46785.full.html#ref-list-1