Distinct Classes of Phosphatidylinositol 3′-Kinases Are Involved in Signaling Pathways That Control Macroautophagy in HT-29 Cells*

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3-Methyladenine which stops macroautophagy at the sequestration step in mammalian cells also inhibits the phosphoinositide 3-kinase (PI3K) activity raising the possibility that PI3K signaling controls the macroautophagic pathway (Blommaart, E. F. C., Krause, U., Schellen, J. P. M., Vreeling-Sindelaróvá, H., and Meijer, A. J. (1997) *Eur. J. Biochem.* 243, 240–246). The aim of this study was to identify PI3Ks involved in the control of macroautophagic sequestration in human colon cancer HT-29 cells. An increase of class I PI3K products (phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate) caused by either feeding cells with synthetic lipids (dipalmitoyl phosphatidylinositol 3,4-bisphosphate and dipalmitoyl phosphatidylinositol 3,4,5-trisphosphate) or by stimulating the enzymatic activity by interleukin-13 reduced macroautophagy. In contrast, an increase in the class III PI3K product (phosphatidylinositol 3-phosphate), either by feeding cells with a synthetic lipid or by overexpressing the p150 adaptor, stimulates macroautophagy. Transfection of a specific class III PI3K antisense oligonucleotide greatly inhibited the rate of macroautophagy. In accordance with a role of class III PI3K, wortmannin (an inhibitor of PI3Ks) inhibits macroautophagic sequestration and protein degradation in the low nanomolar range [IC50 5–15 nM]. Further *in vitro* enzymatic assay showed that 3-methyladenine inhibits the class III PI3K activity. Dipalmitoyl phosphatidylinositol 3-phosphate supplementation or p150 overexpression rescued the macroautophagic pathway in HT-29 cells overexpressing a GTPase-deficient mutant of the Gα3 protein suggesting that both class III PI3K and trimeric Gα3 protein signaling are required in the control macroautophagy in HT-29 cells. In conclusion, our results demonstrate that distinct classes of PI3K control the macroautophagic pathway in opposite directions. The roles of PI3Ks in macroautophagy are discussed in the context of membrane recycling.

Macroautophagy is a major intralysosomal catabolic process conserved during the evolution of eukaryotic cells (1–4). In mammalian cells, the macroautophagic pathway starts with the sequestration of cytoplasmic material (including mitochondria, peroxisomes, smooth and rough membranes, and cytosolic constituents: proteins, glycerol, neutral lipids, RNA, and ribosomes) to form an early autophagosome. The membrane of early autophagosomes is derived from ribosome-free regions of the rough endoplasmic reticulum (5) and/or a related organelle called the phagophore (see Ref. 6 and references therein). After acidification the early autophagosome is transformed in a late autophagosome. Fusion between late autophagosomes and lysosomes triggers the degradation of sequestered material into autolysosomes. Material from the endocytic pathway can be imported in autophagic vacuoles at different steps of their maturation (7, 8).

Recently, studies have shed light on the intracellular mechanisms that control membrane flow along the autophagic/lysosomal pathway in mammalian cells (reviewed in Refs. 9 and 10). It has been suggested that the phosphoinositide 3-kinase (PI3K)* signaling cascade controls macroautophagy (11) and that 3-MA, an inhibitor of macroautophagic sequestration (12), behaves as a PI3K inhibitor.

PI3K belongs to a family of enzymes that phosphorylates the 3′-hydroxy group on the inositol ring of phosphoinositides (reviewed in Refs. 13–16). These phospholipids are involved in a large array of signal transduction pathways controlling mitogenic responses, differentiation, apoptosis, cytoskeletal organization, and membrane flow along the secretory and endocytic pathways (reviewed in Ref. 17). PI3Ks are classified into three classes. Class I enzymes are composed of catalytic p110 subunits and p85 adaptors. The SH2 motif contained in p85 adaptors bind to phosphorylated Tyr residues thereby linking the catalytic subunit to the Tyr kinase signaling pathway (class IA). A member of the class I PI3K family (class IB, p110γ/p101) is activated by subunits of trimeric G proteins (18, 19). Class I PI3Ks phosphorylate PtdIns, PtdIns(4)P, but not PtdIns(4,5)P2. *In vivo* PtdIns(4,5)P2 is likely to be the favorite substrate. Class II enzymes are large enzymes (>200 kDa) characterized by a C-terminal containing a C2 domain (20–22). These enzymes phosphorylate *in vitro* PtdIns, PtdIns(4)P, but not PtdIns(4,5)P2. Class III enzymes

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1The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; diC16PtdIns(3)P, dipalmitoyl phosphatidylinositol 3-phosphate; diC16PtdIns(4)P, dipalmitoyl phosphatidylinositol 4-phosphate; diC16PtdIns(4,5)P2, dipalmitoyl phosphatidylinositol 4,5-bisphosphate; diC16PtdIns(3,4,5)P3, dipalmitoyl phosphatidylinositol 3,4,5-trisphosphate; IL-13, interleukin-13; LDH, lactate dehydrogenase; 3-MA, 3-methyladenine; PKB, protein kinase B, PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; WT, wortmannin; RT-PCR, reverse transcriptase-polymerase chain reaction; SH2, Src homology domain 2.
are homologous to the archetypal Vps34p characterized in Saccharomyces cerevisiae which only produce PtdIns(3)P (24). Vps34p function requires its association with a myristylated serine kinase Vps15p (25). The complex Vps15p-Vps34p is of fundamental importance in controlling vesicular transport to the yeast vacuole (reviewed in Ref. 26). The human homologue of Vps34p has been shown to be associated with a p150 myristylated protein kinase (27).

The goal of the present study was to identify PI3Ks involved in the control of macroautophagy. For this purpose we have used the human colon cancer HT-29 cells which have been demonstrated to be an appropriate model to investigate the signaling pathway that controls macroautophagy (28, 29). In the present study we show that macroautophagic sequestration is distinctly controlled by class I and class III PI3K. Stimulation of class I PI3K activity inhibits macroautophagy at the sequestration step whereas the activity of class III PI3K is required to trigger autophagic sequestration.

**EXPERIMENTAL PROCEDURES**

**Cells and Materials—**HT-29 and Q204L-expressing cells were cultured as previously described (30). HT-29 cells were grown in 100-mm dishes under 10% CO2-air atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin (100 units/ml). 3-MA, phosphoinositides (PtdIns, PtdIns(4)P, and PtdIns(4,5)P2), NADH, metrizamide, and other chemicals were purchased from Sigma. Adenosine was from ICN, histone 2B was from Roche Molecular Biochemicals (Germany). Enzymes, synthetic oligonucleotides, cell culture reagents such as, Geneticin (G418) and LipofectAMINE were from Life Technologies, Inc. (Eragny, France). Nitrocellulose membranes were from Schleicher and Schuell (Dassel, Germany). The phosphoinositides PtdIns(3,4)P2, PtdIns(3,4,5)P3, and rabbit antibodies against p85 and p110α subunits of the class I PI3K were from TEBU (Le Perray en Yvelines, France). Goat anti-PKB polyclonal antibody was from Santa Cruz Biotechnology. The bicinechonic acid (BCA) kit was from Pierce (Rockford, IL). IL-3 and cDNA encoding for the p150 adaptor were kindly provided by A. Minty (Sanofi Recherche, France). Dr. M. D. Waterfield and C. Panaretou (Elf BioRecherche, France) and Dr. M. D. Waterfield and C. Panaretou (Amersham Pharmacia Biotech (Les Ulis, France).

**Autophagic Sequestration of LDH—**Cells were gently washed three times with phosphate-buffered saline (pH 7.4) and lysed with 1 ml of lysis buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM EGTA, and leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 1 mM disodium pyrophosphate, and 1 mM Na2VO4). After preclearing, lysates were immunoprecipitated with goat anti-PKB polyclonal antibody using binding protein A-Sepharose (Amersham Pharmacia Biotech) and analyzed by Western blot using goat anti-PKB to visualize the endogenous protein. Bands were developed by an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) using secondary antibody coupled to horseradish peroxidase.

**Northern Blot Analysis—**The p110α, p85, p150, and villin probes were 32P-labeled to a specific activity of 106 cpm/μg by random priming. Hybridization was performed at 45 °C in the presence of formamide and high stringency washes were performed in 0.1× SSC, 0.1% SDS at 60 °C.

**Reverse Transcription-PCR—**cDNA was synthesized from mRNA isolated from HT-29 cells and was used to amplify a full-length cDNA encoding p110α and class III PI3K subunits, using RT-PCR. The forward primer was a sequence complementary to the 5′ end of the p110α cDNA (5′-ATGCTCTGTGACCTTGTTGATGAG-3′) and the reverse primer was a sequence complementary to the 3′ end of the p110α cDNA (5′-GGATGATGATGATGATGATAA-3′ for p110α, 5′-GACATGATTGCTGCTCTCGTGGAGG-3′ for class III PI3K, 5′-GCAGATTGCTGCTCTGATG-3′ for p150, and 5′-CGATGATTGCTGCTCTGATG-3′ for p150).

**Cell Transfection—**cDNA (1 μg/ml) encoding the p150 was introduced into exponentially growing HT-29 cells by the LipofectAMINE method. Following transfection (48 h), cells were used for different experiments. Phosphorothioate oligonucleotide was synthesized so as to be anticomplementary to the sequence of class III PI3K (5′-TCCCCATGCACGGCCTGTC-3′) (33). The p150 antisense was introduced into exponentially growing HT-29 cells by the LipofectAMINE method. Following transfection cells were used.

**Liposome Formation—**Synthetic phosphatidylinositol phosphate (0.1 mg/ml) and phosphatidylserine (0.1 mg/ml) were solubilized in a mixture of chloroform/methanol, 1:1 (v/v), dried under N2, and dispersed by sonication for 15 min at 20 °C in buffer containing 25 mM HEPES (pH 7.4) and 1 mM EDTA. Samples were centrifuged at 6000 × g for 15 min at 4 °C and the supernatant was added at cells for an incubation of 4 h in a nutrient-free medium.

**PI3K Assay—**PI3K activity was determined by the modified method of Burgeger et al. (34). Briefly, immunoprecipitates and the supernatant of the immunoprecipitation were resuspended in 80 μl of buffer containing 30 mM HEPES and 300 mM adenosine. To both samples, 50 μl of lipid mixture (synthetic lipid and phosphatidylserine) was added for 20 min at 4 °C.

**Measurement of the Degradation of Long-lived Proteins—**Protein degradation was determined as previously reported (30). HT-29 cells were grown on 25-cm2 plates and resuspended in 80 ml of free medium (without amino acids and in absence of fetal calf serum) at the beginning of the chase period. After the first hour of incubation, at which time short-lived proteins were being degraded, the chase medium was replaced with the appropriate fresh medium and the incubation continued for an additional 4 h. Cells were scraped into 0.5 ml of phosphate-buffered saline and the radiolabeled proteins present in the 4-h media and cells were precipitated with 10% trichloroacetic acid. The precipitate was resuspended in the soluble radioactive acid, 1% phosphotungstic acid (v/v) at 4 °C. The precipitate from the control and inhibitor experiments were separated from the soluble radioactivity by centrifugation at 600 × g for 10 min then dissolved in 1 ml of Soluene 350 (Packard) and the associated radioactivity was measured. The rate of protein degradation was calculated as acid soluble radioactivity recovered from both cells and medium. When used, WT, LY294002, synthetic lipids, and 3-MA were added at the beginning of the chase period to maintain PI3K inhibition by WT, we added fresh WT every hour for a total incubation time of 4 h.

**PKB Assay and Western Blots—**PKB activity was determined after immunoprecipitation of HT-29 cell lysates with the goat anti-PKB polyclonal antibody (1/250) using histone 2B as substrate as described previously (32). Briefly, cells grown on 25-cm2 plates were washed once in cold phosphate-buffered saline (pH 7.4) and lysed with 1 ml of lysis buffer (1% Triton X-100, 100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM MgCl2, aprotinin, leupeptin, 1 mM phenylmethylsulfonyl fluoride, 20 mM sodium fluoride, 1 mM disodium pyrophosphate, and 1 mM Na2VO4). After preclearing, lysates were immunoprecipitated with goat anti-PKB polyclonal antibody using binding protein A-Sepharose (Amersham Pharmacia Biotech) and analyzed by Western blot using goat anti-PKB to visualize the endogenous protein. Bands were developed by an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) using secondary antibody coupled to horseradish peroxidase.
mixing and centrifugation, the organic layer was removed, dried under N₂, and resuspended in chloroform/methanol. Extracted lipids were separated by TLC in developing solvents: isopropyl alcohol/H₂O/acetonic acid, 65:34:1 (v:v:v), and then exposed to hyperfilm. Unlabeled standards (1 mg/ml) were revealed by exposure to iodine vapor. Lipids were identified by comparison with unlabeled standards.

RESULTS

WT and LY294002 Inhibit the Macroautophagic Pathway in HT-29 Cells—Recently it has been shown that the PI3K inhibitors WT and LY294002 stop the macroautophagic pathway at the sequestration step in rat hepatocytes (11). In order to investigate the role of PI3K in the control of macroautophagy in HT-29 cells, we have studied the effect of WT and LY294002 on the degradation of [¹⁴C]valine-labeled long-lived proteins (Fig. 1) and on the rate of sequestration of the cytosolic enzyme LDH into autophagic vacuoles (Table I). WT and LY294002 impaired protein degradation in a dose-dependent manner with IC₅₀ values in the range of those previously reported for rat hepatocytes (11). Results reported in Table I show that 10 nM WT and 10 μM LY294002 are as potent as 10 μM 3-MA as inhibitors of autophagic sequestration of the cytosolic enzyme LDH.

Synthetic diC₁₆PtdIns(3,4)P₂, diC₁₆PtdIns(3,4,5)P₃, and IL-13 Inhibit the Macroautophagic Pathway—In order to explore the mechanism by which PI3K controls the macroautophagic pathway, we have first examined the effect of PI3K products administered to HT-29 cells in liposome form using phosphatidylserine as a carrier (see “Experimental Procedures”). Results reported in Fig. 2A, show that diC₁₆PtdIns(3,4)P₂ and diC₁₆PtdIns(3,4,5)P₃ inhibited the degradation of long-lived proteins in a dose-dependent manner. In contrast, diC₁₆PtdIns(4,5)P₂ had no inhibitory effect on protein degradation over the concentration range used. The inhibitory effect of diC₁₆PtdIns(3,4)P₂ and diC₁₆PtdIns(3,4,5)P₃ on the macroautophagic pathway was confirmed by the assay of the sequestration of the cytosolic enzyme LDH (Table I).

The relevance of the effect induced by the synthetic lipids in a cellular environment was studied by measuring the activity of the kinase Akt/PKB which is a well established target of the pleiotropic cytokine IL-13 stimulates PI3K activity and the production of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ in HT-29 cells (38). As shown in Fig. 2B and Table I, IL-13 inhibited the macroautophagic pathway in HT-29 cells. Accordingly stimulation of the PI3K activity by IL-13 (Ref. 38 and data not shown) was associated with the activation of Akt/PKB (Fig. 3).

The bulk of these results seems contradictory because inhibitors and products of PI3K have a similar inhibitory effect on the macroautophagic pathway. However, several classes of PI3K producing different types of phosphoinositides are expressed in the same cell. Thus we next investigated the expression of class I and class III PI3Ks in HT-29 cells. We focused our study on these two families because the class II PI3K does not

![Figure 1](image1.png)

**Fig. 1.** WT and LY294002 inhibit autophagic degradation. The rate of [¹⁴C]valine-labeled long-lived protein degradation was measured in cells incubated in nutrient-free medium as described under “Experimental Procedures.” WT and LY294002 were added at the beginning of the chase period, and fresh WT was added every hour in order to maintain PI3K inhibition. Data are expressed as the percentage of cellular protein degraded during the 4-h period of chase. The values reported are the means of five determinations ± S.D.

![Table I](image2.png)

**Table I**

| Treatment | Autophagic sequestration of LDH |
|-----------|--------------------------------|
|           | %/h                             |
| Untreated cells | 4.0 ± 0.6*                 |
| 3-MA (10 mM)   | 1.6 ± 0.3                  |
| WT (10 nM)     | 1.7 ± 0.3                  |
| LY294002 (10 μM) | 1.5 ± 0.2               |
| IL-13 (10 μM)  | 2.0 ± 0.2                  |
| PtdIns(3)P₂ (10 μM) | 6.5 ± 0.7             |
| PtdIns(4)P₂ (10 μM) | 3.5 ± 0.2             |
| PtdIns(3,4)P₂ (10 μM) | 2.0 ± 0.1             |
| PtdIns(4,5)P₂ (10 μM) | 3.7 ± 0.5             |
| PtdIns(3,4,5)P₃ (10 μM) | 2.0 ± 0.2            |

* Values are the mean ± (S.D.) (n = 5).

![Figure 2](image3.png)

**Fig. 2.** Class I PI3K products and IL-13 inhibit the autophagic pathway. Dose-response of diC₁₆PtdIns(3,4)P₂ (●), diC₁₆PtdIns(3,4,5)P₃ (△), diC₁₆PtdIns(4,5)P₂ (□) panel A), and IL-13 (panel B) on autophagic degradation. The different compounds were added at the beginning of the chase period in nutrient-free medium. Data are expressed as the percentage of cellular protein degraded during the 4-h period of chase. The values reported are the means of five determinations ± S.D.

![Figure 3](image4.png)

**Fig. 3.** IL-13 and diC₁₆PtdIns stimulate activation of the PI3K effector PKB. HT-29 cells were treated with IL-13 (30 ng/ml), diC₁₆PtdIns(3,4)P₂, or diC₁₆PtdIns(4,5)P₂ at 10 μM for the chase period. Cells were then lysed and immunoblotted with a PKB antibody. For the assay, immunocomplexes were subjected to an in vitro kinase assay, using histone 2B as substrate (see “Experimental Procedures”). The upper panel represents the phosphorylation status of histone 2B and the lower panel is a Western blot analysis of Akt/PKB protein. Data are from a single experiment representative of at least three others.

diC₁₆PtdIns(3,4,5)P₃. It has been demonstrated that the pleiotropic cytokine IL-13 stimulates PI3K activity and the production of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ in HT-29 cells (38). As shown in Fig. 2B and Table I, IL-13 inhibited the macroautophagic pathway in HT-29 cells. Accordingly stimulation of the PI3K activity by IL-13 (Ref. 38 and data not shown) was associated with the activation of Akt/PKB (Fig. 3).
produce PtdIns(3,4,5)P$_3$ from PtdIns(4,5)P$_2$ and the widely expressed class II isoform PI3K-C2a is poorly sensitive to WT (13, 22, 23). These characteristics make the involvement of class II PI3K in the control of macroautophagy unlikely.

Characterization of PI3Ks Expressed in HT-29 Cells—RT-PCR and Northern blot analyses were used to characterize the different classes of PI3K present in HT-29 cells (Fig. 4A). Two different mRNAs of catalytic subunits of PI3K were detected corresponding to the class I p110$\alpha$ and the class III PI3K, respectively. The presence of other class I PI3K catalytic subunits was not considered.

Class I and class III PI3Ks were also identified by their enzymatic activities (Fig. 4B). After specific immunoprecipitation of the class I PI3K from HT-29 homogenates enzymatic activities were determined in the pellet (class I activity) and supernatant (class III activity). Using PtdIns(4,5)P$_2$ as a substrate we observed production of PtdIns(3,4,5)P$_3$ in the pellet derived from IL-13-treated cells whereas the basal activity of class I PI3K was very low in unstimulated HT-29 cells (Fig. 4B). In the supernatant, using PtdIns as a substrate, we observed the production of PtdIns(3)P when Mn$^{2+}$ was present in the assay whereas the activity was barely detectable when Mg$^{2+}$ was used instead of Mn$^{2+}$ (data not shown). This cation dependence has been shown to be a hallmark of class III PI3K (39). Furthermore, and in contrast to the class I PI3K, the activity of class III PI3K was not dependent on IL-13 treatment.

The Class III PI3K Product Stimulates the Macroautophagic Pathway—In order to determine the role of class III PI3K in the control of macroautophagy, we have tested the effect of PtdIns(3)P, the only product of class III PI3K (24, 39). For this purpose, autophagic parameters were determined in cells fed with diC16PtdIns(3)P (Fig. 5 and Table I). Results reported on Fig. 5A show that diC16PtdIns(3)P-stimulated protein degradation in a dose-dependent manner whereas its isomer diC16PtdIns(4)P had no effect on protein degradation. According to these data diC16PtdIns(3)P but not diC16PtdIns(4)P also stimulated autophagic sequestration (Table I). Moreover, diC16PtdIns(3)P was able to reverse the inhibitory effect of WT on protein degradation (Fig. 5C).

The role of class III PI3K in the control of macroautophagy was further investigated using an antisense approach. Oligonucleotides were selected as described previously (33). After introducing antisense oligonucleotides the class III PI3K activity was inhibited by 90% (Fig. 6A) whereas the activity of class I PI3K was not modified (data not shown). In antisense expressing cells we observed an inhibition of long-lived protein degradation when compared with untransfected cells (Fig. 6B).

The p150 Adaptor Stimulates Class III PI3K-dependent Macrautophagy—In mammalian cells, class III PI3K is associated with a p150 membrane adaptor. This adaptor allows the recruitment of class III PI3K to membranes and stimulates its lipid kinase activity (27). In order to investigate whether the complex p150/class III PI3K is required to stimulate the macroautophagic pathway we have transfected HT-29 cells with the cDNA encoding p150. As determined by Northern blot, the expression of p150 was increased in transfected cells (Fig. 7A) and the activity of class III PI3K was also increased by 2.5 times (Fig. 7B). Accordingly, the rate of long-lived protein degradation was stimulated in cells overexpressing the p150 adaptor (Fig. 7C). These results suggest that the inhibition of class III PI3K by WT or 3-MA could be responsible for the interruption of the macroautophagic pathway. In order to investigate this possibility we have analyzed the effect of 3-MA on the class III PI3K activity, in vitro (Fig. 7D). The production of PtdIns(3)P was inhibited in a dose-dependent manner by 3-MA with a maximal inhibition of 85–90% when 10 mM 3-MA was used (Fig. 7D).

Class III PI3K and the Heterotrimeric G$_{i3}$ Protein Signaling Pathways Are Required to Control Macroautophagy in HT-29 Cells—We have previously reported that in HT-29 cells the macroautophagic pathway is controlled at the sequestration step by the activity of a cytoplasmic heterotrimeric G$_{i3}$ protein (28). Autophagic sequestration is inhibited when the G$_{i3}$ protein is bound to GTP. This conclusion was reached by introducing a point mutation at position 204 in the G$_{i3}$ protein to generate the Q204L G$_{i3}$ protein. Cells transfected with the cDNA encoding the Q204L G$_{i3}$ protein have a very low rate of macroautophagic degradation when compared with untransfected HT-29 cells (30).

In order to investigate the respective role of the G$_{i3}$ protein and the class III PI3K in the control of macroautophagy we have fed Q204L-expressing cells with synthetic phosphoinositides (diC16PtdIns(3)P and diC16PtdIns(4)P). Of the lipids used only diC16PtdIns(3)P stimulated long-lived protein degradation (Fig. 5B) and reversed the inhibition of the macroautophagic pathway in Q204L-expressing cells in the presence or absence of WT (Fig. 5D). However, the rate of protein degra-
The rate of \[^{14}C\]valine-labeled long-lived protein degradation was measured in cells incubated in a nutrient-free medium. In panels C and D, WT (10 nM) was added together with diC16PtdIns(3)P (10 μM) during the 4-h chase period. Data are expressed as the percentage of cellular protein degradation during the 4-h chase period. The values reported are the means of five determinations ± S.D.

**DISCUSSION**

In this report we show that WT and LY294002, two PI3K inhibitors, stop the macroautophagic pathway at the sequestration step in HT-29 cells. These results confirm previous data obtained in rat hepatocytes (11) and support the notion that PI3Ks control macroautophagy in mammalian cells. According to these data, 3-MA, a long known macroautophagic sequestration inhibitor (12), is also a PI3K inhibitor (Ref. 11 and this study). The overlapping inhibitory profile of 3-MA and WT explains the observation that both drugs interrupt transport from late endosomes to lysosomes (40, 41).

Here we report data showing that two different classes of PI3Ks control the macroautophagic sequestration. Class I PI3Ks and class III PI3Ks have distinct effects on the macroautophagic sequestration. The class I synthetic products (diC16PtdIns(3,4)P₂ and diC16PtdIns(3,4,5)P₃) inhibited both the macroautophagic sequestration and degradation. Although the mechanism by which these lipids are incorporated into cells is unknown, they maybe at least incorporated into the cell membrane where they are able to activate Akt/PKB. The inhibitory effect of the synthetic lipids used are specific since diC16PtdIns(4,5)P₂ cannot substitute diC16PtdIns(3,4)P₂. Macroautophagy was also inhibited when class I PI3K was activated by IL-13 treatment. Similarly to other cytokines such as IL-2 and IL-3 (42–44), the IL-13 transduction pathway activates Akt/PKB (Ref. 45 and this study). Nevertheless our results provide no direct evidence that the activation of Akt/PKB is required for controlling macroautophagic sequestration. Further studies are needed to investigate the role of Akt/PKB in macroautophagy and its link with the activation of p70S6 kinase, a possible downstream target of Akt/PKB (46), which is a key event in the inhibition of macroautophagic sequestration (47). However, other possibilities must be considered to explain the role of class I PI3K. Recently it has been demonstrated that class IA PI3K can be translocated onto the endoplasmic reticulum membrane (48, 49) which is the most likely origin of nascent autophagic vacuole membrane (5). Interestingly, class IA PI3K is associated with the ER membrane in rat hepatocytes after insulin stimulation or refeeding (48, 49), two conditions known to reduce the macroautophagic pathway (4).
The activity of Fab1p PtdIns(3)P 5-kinase is essential to maintain the vacuolar size and membrane homeostasis (56). Similarly to the yeast model, the availability of PtdIns(3,5)P2 has been shown to regulate the production of PtdIns(3,5)P2 in fibroblasts (57). As recently hypothesized for yeast, the production of PtdIns(3,5)P2 in mammalian cells could control the membrane recycling of post-Golgi membranes (56). Accordingly, stimulation of class III PI3K by p150 overexpression and feeding WT-treated cells with diC16PtdIns(3)P would favor the production of PtdIns(3,5)P2 and membrane recycling. This membrane recycling would then control the de novo autophagic vacuole formation. In contrast, WT or 3-MA treatment which inhibit the formation of PtdIns(3,5)P2 would reduce the synthesis of PtdIns(3,5)P2 and consequently perturb membrane recycling. This hypothesis is in line with the pleiotropic effect of WT on multivesicular bodies and Golgi homeostasis (58, 59). The relation between the production of PtdIns(3,5)P2 and the formation of autophagic vacuoles is now under investigation in our laboratory.

Previously we have shown that macroautophagy is controlled by a trimeric Gα3 protein (28, 30). Our results (Fig. 7B) show that the activity of class III PI3K is similar in HT-29 cells and in cells expressing a GTPase-deficient mutant of the Gα3 protein (Q204L-expressing cells) with a low rate of macroautophagy (30). This observation suggests that both Gα3 and PI3K signaling pathways are required to control macroautophagy or that class III PI3K acts upstream the Gα3 protein. However, the observation that feeding with diC16PtdIns(3)P or overexpression of the p150 adaptor partially rescues autophagy in Q204L-expressing cells suggests that both signaling pathways are required to control autophagic sequestration. Possibly Gα3 acts on the degradation of the PtdIns(3)P lipids.

In conclusion, our data show that macroautophagy in HT-29 cells is controlled by class III and class I PI3K in opposite directions. While the product of class III PI3K stimulates macroautophagy, the products of class I PI3K inhibit this process.

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FIG. 7. p150 adaptor stimulates the class III PI3K-dependent macroautophagy. HT-29 cells and Q204L-expressing cells were transfected with p150 cDNA by the LipofectAMINE method. Panel A, Northern blot analyses were done on both untransfected and transfected cells, in order to determine the level of p150 mRNA. Panel B, values of PI3K activity class III in p150 transfected and untransfected cells. Panel C, effect of [14C]valine-labeled long-lived protein degradation in cells transfected with the cDNA encoding p150. Panel D, effect of WT and 3-MA on class III PI3K activity in HT-29 cells. Increasing concentrations of 3-MA were added at the beginning of the kinase assay. The symbol &oplus; on the ordinate axis represents the class III PI3K activity measured in the presence of 10 nM WT. Class III PI3K activity was performed in the presence of 100 μM ATP. The values reported are the means of 3 determinations ± S.D.
3'-Phosphorylated Phosphoinositides in Macroautophagy