Distinct Roles for the p110α and hVPS34 Phosphatidylinositol 3’-Kinases in Vesicular Trafficking, Regulation of the Actin Cytoskeleton, and Mitogenesis

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Abstract. We have examined the roles of the p85/p110α and hVPS34 phosphatidylinositol (PI) 3’-kinases in cellular signaling using inhibitory isoform-specific antibodies. We raised anti-hVPS34 and anti-p110α antibodies that specifically inhibit recombinant hVPS34 and p110α, respectively, in vitro. We used the antibodies to study cellular processes that are sensitive to low-dose wortmannin. The antibodies had distinct effects on the actin cytoskeleton; microinjection of anti-p110α antibodies blocked insulin-stimulated ruffling, whereas anti-hVPS34 antibodies had no effect. The antibodies also had different effects on vesicular trafficking. Microinjection of inhibitory anti-hVPS34 antibodies, but not anti-p110α antibodies, blocked the transit of internalized PDGF receptors to a perinuclear compartment, and disrupted the localization of the early endosomal protein EEA1. Microinjection of anti-p110α antibodies, and to a lesser extent anti-hVPS34 antibodies, reduced the rate of transferrin recycling in CHO cells. Surprisingly, both antibodies inhibited insulin-stimulated DNA synthesis by 80%. Injection of cells with antisense oligonucleotides derived from the hVPS34 sequence also blocked insulin-stimulated DNA synthesis, whereas scrambled oligonucleotides had no effect. Interestingly, the requirement for p110α and hVPS34 occurred at different times during the G1–S transition. Our data suggest that different PI 3’-kinases play distinct regulatory roles in the cell, and document an unexpected role for hVPS34 during insulin-stimulated mitogenesis.

Key words: phosphatidylinositol 3’-kinase • signal transduction • endocytosis • vesicular trafficking • phosphoinositides
contain specialized lipid-binding domains (Lemmon et al., 1996). Examples include the serine kinase Akt/PKB and its upstream activator the 3-phosphoinositide-dependent kinase-1, which contain Pleckstrin homology domains that bind PI[3,4]P2 and PI[3,4,5]P3 (for review see Toker and Cantley, 1997), and the endosomal protein EEA1, which contains a zinc finger domain that binds to PI[3]P (Stenmark et al., 1996; Patki et al., 1997).

The functions of the different PI 3'-kinases in mammalian cells have been studied using tools that vary significantly in their specificity. Mutant p85 molecules or SH2 domains from p85 produce dominant-negative phenotypes in microinjected or transfected cells (Jhun et al., 1994; Hará et al., 1995), and mutagenesis of p85 binding sites in receptor tyrosine kinases blocks p85/p110 activation (for review see Cantley et al., 1991). However, these approaches do not distinguish between different p110 isoforms which should all bind p85. Less-specific approaches include overexpression of the p110α PI 3'-kinase, which produces PI[3]P, PI[3,4]P2, and PI[3,4,5]P3 and can feed into pathways normally regulated by class I, II, or III enzymes. Similarly, treatment of cells with wortmannin inhibits both the mammalian class I and class III enzymes at low nanomolar doses (Vanhaesebroeck et al., 1997). Unlike the mammalian enzyme, the yeast VPS34 is relatively insensitive to wortmannin [Schu et al., 1993]) The use of wortmannin is further complicated by the fact that inhibition of mammalian class II PI 3'-kinases requires high nanomolar to micromolar concentrations (Domin et al., 1997). These doses also inhibit PI 4'-kinases, and could affect cellular levels of PI[4,5]P2 (Meyers and Cantley, 1997).

The p85/p110 PI 3'-kinases have been implicated in mitogenic signaling, regulation of the actin cytoskeleton, resistance to apoptosis, and trafficking of the Glut 4 glucose transporter (Fruman et al., 1998). In contrast, little is known about the function of class III PI 3'-kinases in mammalian cells. In S. cerevisiae and S. pombe, VPS34-null strains show disruption of vacuolar sorting at permissive and nonpermissive temperatures, and reduced growth at elevated temperatures (Herman and Emr, 1990; Takegawa et al., 1995). The authors suggest that the combined stress of high temperature plus abnormal vacuolar function may inhibit growth at high temperature. Alternatively, they suggest that some vacuolar function might be required for high temperature growth (Takegawa et al., 1995). In D. discoideum, reduced expression of the DdPIK5 homologue leads to reduced growth on bacterial lawns but not in suspension culture (Zhou et al., 1995), suggesting a lysosomal defect that causes a reduced ability to utilize bacteria as food. On the other hand, a complete gene knockout of the D. discoideum VPS34 homologue is lethal, implying that VPS34 may be essential for growth.

Our approach has been to study the intracellular function of distinct PI 3'-kinase by developing isoform-specific inhibitory anti-PI 3'-kinase antibodies. We have focused on the role of p110α and hVPS34, which are wortmannin-sensitive class I and III enzymes, in a number of wortmannin-sensitive responses. We find that insulin-stimulated reorganization of filamentous actin is inhibited by microinjection of antibodies to p110α but not hVPS34. In contrast, antibodies to both enzymes inhibit vesicular trafficking: anti-hVPS34 antibodies interfere with the sorting of endocytosed PDGF receptors, disrupt the localization of the early endosomal protein EEA1, and modestly inhibit transferrin recycling, whereas anti-p110α antibodies strongly inhibit transferrin recycling. Surprisingly, antibodies to hVPS34 as well as p110α inhibit insulin-stimulated DNA synthesis. However, the requirement for p110α and hVPS34 occur at different times during the G1–S transition. These studies represent a first step in the assignment of distinct PI 3'-kinases to the regulation of distinct cellular events.

Materials and Methods

Cells

Growth of GRC-LR+73 cells, an insulin-responsive derivative of CHO cells, has been previously described (Pollard and Stanners, 1979; McIlroy et al., 1997). Hep G2 cells expressing the wild-type PDGF receptor (Va- lius and Kazlauskas, 1995), C6 cells (Harvard University, Cambridge, MA) and were grown in DMEM containing 10% fetal bovine serum. Trvb-1 cells, a CHO cell line expressing the human transferrin receptor (McGrath et al., 1987), were generously provided by T. McGrath (Cornell University School of Medicine, New York, NY) and were grown in α MEM containing 10% fetal bovine serum.

Antibodies

Anti-hVPS34 antibodies were raised in New Zealand white rabbits against a peptide corresponding to residues 571–887 of the human VPS34 sequence, AVVEQHHKRAQYWRK (Volinia et al., 1995). Antibodies were purified using an affinity column made from the same peptide coupled to CNBr Sepharose (Pharmacia Biotech, Piscataway, NJ). Antibodies for microinjection were dialyzed into phosphate-buffered saline and concentrated to 3 mg/ml. Antibodies to p110α have been previously described (McIlroy et al., 1997). Anti-EEA1 antibodies were purchased from Transduction Laboratories (Lexington, KY).

Antisense Oligonucleotides

Phosphorothioate oligonucleotides were synthesized (Genelink, Thornwood, NY) so as to be anticomplementary to sequences from hVPS34. AS1: TCCCCATTCTCTGCTTCCCCCAT (based on nucleotides 36–56 in the hVPS34 GenBank/EMBL/DDBJ sequence Z46973); AS2: AAACTTGCTCTGCTTCCCCCAT (based on nucleotides 48–68); AS3: TCTGATCATCATGTCAT (based on nucleotides 491–511).

Production of Recombinant p110α and hVPS34

Sf-9 cells were infected with recombinant baculovirus for bovine p110α (cDNA provided by M. Waterfield, Ludwig Institute for Cancer Research, London, UK), hVPS34 (virus provided by M. Waterfield) or p110β (virus provided by A. Morris, State University of New York at Stony Brook, Stony Brook, NY). After 2 d in culture, the cells were washed in ice-cold PBS and lysed by freeze-thawing in 10 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 100 µg/ml aprotinin, 1 µg/ml leupeptin, and 350 µg/ml PMSF. After removal of particulate material by centrifugation at 12,000 × g, the lysates were assayed directly for PI 3'-kinase activity as described below.

In Vitro PI 3'-Kinase Assays

Sf-9 lysates containing recombinant p110α, p110β, or hVPS34 were incubated in the absence or presence of antibodies as described in the text. PI 3'-kinase activity was then assayed using sonicated bovine liver phosphatidyl-}

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Cells were incubated for 5 h in cysteine/methionine-free medium containing 1 mCi/ml \[^{35}S\]cysteine/methionine (Easy-tag, New England Nuclear, Boston, MA). The cells were then washed with phosphate-buffered saline, lysed in under non-denaturing (Yu et al., 1998) or denaturing (Martys et al., 1996) conditions, and then proteins were immunoprecipitated with anti-hVPS34 or anti-p110α antibodies and absorbed to protein A-Sepharose beads. The beads were washed five times in RIPA buffer, followed by an additional four washes in RIPA containing 1 M NaCl. After a final wash in PBS, the proteins were eluted, separated by SDS-PAGE, and then visualized by autoradiography.

**Microinjection**

Cells were grown on polylysine-coated glass coverslips. GRC-LR+73 cells were transferred to medium containing 1% fetal bovine serum for 48 h before injection. HepG2/PDGF-R cells were incubated in serum-free medium overnight before injection. Microinjections were conducted using an Eppendorf semiautomated microinjection system and needles pulled on a Sutter p-87 micropipette puller. Antibodies (2–4 mg/ml) or oligonucleotides (10 μM) were mixed with nonspecific rabbit IgG in PBS, pH 7.4, to a final antibody concentration of 3 mg/ml. Cells were allowed to recover for 2 h before further manipulation.

**Actin Reorganization**

GRC-LR+73 cells were injected with control or anti-PI 3'-kinase antibodies as indicated. After a 2-h recovery period, the cells were stimulated with insulin for 7 min, fixed with 3.7% formaldehyde, and stained with FITC anti-rabbit antibodies (to detect injected cells) or rhodamine-phalloidin (Molecular Probes, Eugene, OR), to visualize the actin cytoskeleton (Segall et al., 1996).

**EEA1 Staining**

Cells were injected with control IgG or anti-PI 3'-kinase antibodies, allowed to recover for 2 h, and then fixed with 3.7% formaldehyde for 20 min on ice. The cells were permeabilized with methanol on dry ice, blocked with 10% goat serum, and stained with FITC anti-rabbit IgG (to detect injected cells) or anti-EEA1 antibodies (Transduction Laboratories). EEA1 staining was visualized using the Renaissance signal amplification kit (New England Nuclear Life Science Products, Boston, MA), according to the manufacturer’s instructions.

**Transferrin Recycling**

Trvb-1 cells were injected with control or anti-PI 3'-kinase antibodies as indicated, and allowed to recover for 2 h. The cells were loaded with Cy3-transferrin for 2 h, washed, and then fixed immediately or after an additional hour in transferrin-free medium as previously described (Martys et al., 1996). The cells were permeabilized with saponin as described (McGraw et al., 1987) and stained with FITC anti-rabbit antibodies to visualize injected cells.

**PDGF Receptor Trafficking**

PDGF receptor internalization was measured as described by Joly et al. (1994). HepG2/PDGF-R cells were incubated in serum-free medium overnight. The cells were injected with control or anti-PI 3'-kinase antibodies and allowed to recover for 2 h. The cells were then incubated for 70 min on ice with monoclonal anti-PDGF receptor antibody (20 μg/ml final; Calbiochem-Novabiochem, La Jolla, CA) and recombinant human PDGF-BB (20 ng/ml; Calbiochem-Novabiochem) for 2 h. The cells were then fixed with FITC anti-rabbit antibodies to visualize injected cells, or Cy3–anti-mouse antibodies to visualize PDGF receptors.

**BrdU Incorporation**

After injection, cells were kept in medium containing 1% FBS or stimulated with 100 nM insulin as indicated. When indicated, the cells were injected at various times after insulin stimulation. 16 h after the onset of insulin stimulation, the cells were incubated with 100 μM BrdU for 2 h and fixed in 3.7% formaldehyde. Nuclear DNA was denatured by treating the cells with 4 N HCl for 3 min, and the cells were permeabilized in methanol at −20°C and stained with rhodamine-conjugated anti-BrdU antibody to measure DNA synthesis, and FITC-conjugated anti-rabbit IgG to determine microinjected cells. The percentage of microinjected cells that was positive for BrdU staining was determined. Data from each experiment reflects the counting of ~100 injected cells per condition. The mean and SEM values were generated by pooling percentages from different experiments, where n = the number of separate experiments.

**Results**

**Characterization of Anti-p110α and Anti-hVPS34 Antibodies**

We have previously described antibodies to residues 1054–1068 of p110α, which inhibit p110α in vitro and in microinjected cells (McIlroy et al., 1997). We also raised antibodies against residues 871–887 at the COOH terminus of the human VPS34 primary sequence. To test the specificity of the antibodies, we labeled three different cell lines for 5 h with a mixture of \[^{35}S\]methionine and \[^{35}S\]cysteine. The lines were: a CHO-derived line selected for tight quiescence during serum withdrawal (Pollard and Stanners, 1979) (GRC-LR+73), a CHO line expressing the human transferrin receptor (McGraw et al., 1987) (Trvb-1), and a HepG2 line expressing the human PDGF receptor (Valius and Kazlauskas, 1993). We then lysed the cells under non-

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denaturing and denaturing conditions, and performed immunoprecipitations with control IgG or the anti-PI kinase antibodies. The anti-p110α antibodies were ineffective under denaturing conditions, but under non-denaturing conditions precipitated a single 110-kD band from all three cell lines (Fig. 1, left). The p85 regulatory subunit of p85/p110 PI 3'-kinase was not observed in these experiments, presumably because p110α has significantly more cysteine and methionine residues (70, versus 16 for p85) and turns over more rapidly than p85 (Yu et al., 1998). The anti-hVPS34 antibodies was effective under denaturing conditions and precipitated a major specific 100-kD band from all three cell lines (Fig. 1, right). Additional bands minor were observed but were also present after immunoprecipitation with control IgG, and therefore reflect nonspecific interactions.

We further characterized the new anti-hVPS34 antibodies. They could immunoprecipitate recombinant human hVPS34 from SF-9 lysates (Fig. 2 A). The antibody also precipitate a PI 3'-kinase activity from CHO lysates; this activity was manganese dependent, consistent with the ion specificity of hVPS34 (Fig. 2 B) (Volinia et al., 1995). Although we could measure PI kinase activity in the anti-hVPS34 immunoprecipitates, the antibody was in fact inhibitory toward hVPS34. Fig. 2 C shows that the activity of immunoprecipitated recombinant hVPS34 was increased when eluted from antibody–protein A beads by incubation with the antigen peptide. Inhibition of hVPS34 could be directly measured in a soluble in vitro assay with recombinant hVPS34, hVPS34 activity was inhibited 75% by anti-hVPS34 antibodies, but was unaffected by a previously described antibody that binds and inhibits the p110α PI 3'-kinase (Fig. 2 D, left). Importantly, the anti-hVPS34-1 antibody had no effect on the activity of recombinant p110α (Fig. 2 D, right). In contrast, anti-p110α antibody inhibited p110α by 80%. Neither antibody inhibited the activity of recombinant p110β (data not shown). Inhibition of hVPS34 by the anti-hVPS34 antibody was dose dependent (Fig. 2 E).

**Role of p110α and hVPS34 in Insulin-stimulated Membrane Ruffling**

We used the isoform-specific antibodies to examine the roles of p110α and hVPS34 in the regulation of the actin cytoskeleton. GRC-LR+73 cells were microinjected with control IgG or anti-PI kinase antibody, incubated in the absence or presence of 100 nM insulin for 7 min, and then fixed. Cells were stained with FITC-labeled anti-rabbit antibodies to identify microinjected cells (Fig. 3, left). In quiescent cells injected with anti-hVPS34 antibodies (Fig. 3, A and B), quiescent cells injected with anti-hVPS34 antibodies (Fig. 3, E and F) or anti-p110α antibodies (Fig. 3, I and J) were similar to the IgG-injected cells in appearance. When IgG-injected cells were stimulated with insulin for 7 min, prominent actin-rich projections appeared at the periphery of the cells (Fig. 3, C and D). These projections appear somewhat blurry, as they extend out of the plane of focus used to visualize the stress fibers. In cells injected with anti-hVPS34 antibodies, insulin-stimulation of actin-rich projections was also apparent (Fig. 3, G and H). However, injection of cells with antibodies against p110α completely blocked the insulin-stimulated appearance of actin-rich projections (Fig. 3, K and L). These data implicate p110α, rather than hVPS34, as critical for insulin-stimulated rearrangement of the actin cytoskeleton.
Figure 3. Insulin regulation of the actin cytoskeleton requires p110α but not hVPS34. Quiescent GRC-LR+73 cells were injected with 3 mg/ml rabbit IgG (A–D), anti-hVPS34 (E–H) or anti-p110α (I–L). The cells were fixed in the absence (A, B, E, F, I, and J) or presence (C, D, G, H, K, and L) of 100 nM insulin for 7 min. The cells were fixed and stained with FITC anti-rabbit IgG (left) or rhodamine-phalloidin (right). Asterisk, injected cells on the right. Images were collected using a Nikon Diaphot inverted microscope with Nikon 100× NA 1.25 Achromat optics. The data is representative of two experiments.

Role of p110α and hVPS34 in Early Endosomal Events

Recent data from Corvera, Stenmark, and their colleagues have shown that the early endosomal protein EEA1 is displaced from endosomes by wortmannin and binds specifically to vesicles containing PI[3]P (Patki et al., 1997, 1998; Gaullier et al., 1998). Although the production of PI(3)P in the endosomal membrane could be the result of any of the known PI 3'-kinases, the exclusive production of PI(3)P by hVPS34 would make it a likely candidate. We therefore tested the effects of the inhibitory antibodies on the localization of EEA1. In control Trvb-1 cells or cells injected with rabbit IgG (Fig. 4 A), EEA1 is located in large vesicular structures scattered throughout the cytoplasm. Injection of Trvb-1 cells with anti-p110α antibodies (Fig. 4 B) had little effect on EEA1. In contrast, in cells injected with anti-hVPS34 antibodies, EEA1 was no longer associated with small vesicles but concentrated in large perinuclear structures (Fig. 4 C). This change in the subcellular localization of EEA1 was similar to that seen in Trvb-1 cells treated with 100 nM wortmannin (Fig. 4, D and E). These data suggest that hVPS34 is required for the localization of EEA1, presumably by producing PI(3)P in the early endosomal membrane.

We also examined the postendocytic sorting of internalized PDGF receptors to a perinuclear compartment, which is blocked by wortmannin (Joly et al., 1995). HepG2 cells expressing the wild-type PDGF receptor were incubated at 4°C with mouse anti-PDGF receptor antibodies and 20 ng/ml PDGF, then rapidly warmed to 37°C for 10 min to initiate endocytosis. After fixation, injected cells were visualized with FITC-labeled anti-rabbit antibodies (Fig. 5, left) and PDGF receptors were visualized with Cy3-labeled...
anti-mouse antibodies (Fig. 5, right; asterisks, injected cells). In cells that were fixed before the 10 min incubation at 37°C, anti-PDGFR receptor staining was entirely in the plasma membrane and was unaffected by injection with control or anti-PI 3'-kinase antibodies (data not shown). In cells that were warmed to 37°C for 10 min, internalized receptors accumulated in small peripheral vesicles as well as a prominent ring of larger perinuclear vesicles (Fig. 5 B). This perinuclear accumulation of PDGF receptors was not affected by injection with control IgG (Fig. 5, A and B) or anti-p110α antibodies (Fig. 5, C and D). In both cases, the distribution of PDGF receptors in injected cells is the same as that seen in noninjected cells in the same field. In contrast, injection of cells with inhibitory anti-hVPS34 antibodies almost completely blocked the perinuclear accumulation of internalized PDGF receptors (Fig. 5, E and F). The staining in these cells was similar to that seen in cells treated with 100 nM wortmannin (data not shown).

The effects of inhibitory anti-hVPS34 antibodies on the sorting of PDGF receptors was more clearly seen in cells fixed after 5 min at 37°C (Fig. 6). In control cells or cells injected with rabbit IgG (Fig. 6 A), PDGF receptors could be seen in small vesicles scattered throughout the cytoplasm, as well as large vesicles near the nucleus. Injection of anti-hVPS34 antibodies (Fig. 6 B) does not block internalization of PDGF receptors, but the internalized receptors are located in smaller vesicles that are primarily located at the cell periphery. These data would be consistent with a role for hVPS34 in the fusion and/or maturation of early endosomal vesicles.

Role of p110α and hVPS34 in Transferrin Recycling

We next examined the recycling of endocytosed transferrin, which is significantly slowed in cells treated with 100 nM wortmannin (Martys et al., 1996; Spiro et al., 1996). Trvb-1 cells were injected with control IgG or anti-PI kinase antibodies, labeled for 2 h with Cy3-transferrin, and washed into transferrin-free medium. We visualized the injected cells with FITC-labeled anti-rabbit IgG (Fig. 7, left) and compared their content of Cy3 transferrin (Fig. 7, right). None of the antibodies affected the steady state labeling of CHO cells, observed after the 2-h incubation with Cy3-transferrin (Fig. 7, A, C, and E); the internalized transferrin accumulated in a diffuse perinuclear compartment as previously described (McGraw et al., 1987; Martys et al., 1996). After 60 min in transferrin-free medium, most of the internalized Cy3-transferrin had effluxed from cells injected with control IgG (Fig. 7 B). Cells injected with anti-hVPS34 antibodies showed a slightly higher level of residual Cy3-transferrin after 60 min (Fig. 7 D). In contrast, cells injected with anti-p110α antibodies showed a persistent labeling with Cy3 after the 60-min incubation in transferrin-free medium (Fig. 7 F), reflecting a delay in the efflux of internalized Cy3-transferrin. These data are similar to those obtained from cells treated with 100 nM wortmannin (Martys et al., 1996), and suggest that p110α is the primary wortmannin-sensitive PI 3'-kinase involved in regulation of transferrin recycling.

A 60-min chase in transferrin-free medium was chosen to maximize the differences between cells injected with
Distinct Functions of \( p110 \alpha \) and \( hVPS34 \) PI Kinases

Figure 5. PDGF receptor trafficking requires \( hVPS34 \), but not \( p110 \alpha \). HepG2 cells expressing the human PDGF-\( \beta \)-receptor were injected with 3 mg/ml rabbit IgG (A and B), anti-\( p110 \alpha \) (C and D) or anti-\( hVPS34 \) (E and F). The cells incubated at 4°C with anti-PDGF receptor antibodies and 20 ng/ml PDGF, warmed rapidly to 37°C for 10 min, and then fixed. The cells were stained with FITC anti-rabbit IgG to labeled injected cells (left) or Cy3-anti-mouse IgG to label PDGF receptors (right). Asterisk, injected cells on the right. The data is representative of six experiments. Images were collected using a Nikon Diaphot inverted microscope with Nikon 100× NA 1.25 Achromat optics.

Role of \( p110 \alpha \) and \( hVPS34 \) in Insulin-stimulated Mitogenic Signaling

Roche et al. (1994) and ourselves have previously shown that inhibitory antibodies to \( p110 \alpha \) block mitogen-stimulated DNA synthesis. To compare the role of \( p110 \alpha \) and \( hVPS34 \) in mitogenic signaling, GRC-LR+73 cells were injected with control IgG or anti-PI 3’-kinase antibodies, stimulated with insulin for 16 h and labeled with BrdU. Surprisingly, injection of anti-\( hVPS34 \) as well as anti-\( p110 \alpha \) antibodies inhibited insulin-stimulated DNA synthesis by 70% (Fig. 8).

To confirm the unexpected requirement for \( hVPS34 \) in mitogenic signaling, we designed three antisense phosphorothioate oligonucleotides derived from NH₂-terminal or internal nucleotide sequences from \( hVPS34 \) (Materials and Methods). We initially tested these oligonucleotides in HeLa cells, as they were derived from a human sequence and were more likely to work in a human line. HeLa cells were difficult to render quiescent; after 2 d without serum the cells were still 28% BrdU positive, and insulin stimulation caused only a twofold increase in BrdU labeling to 58%. Nonetheless, this increase was completely blocked by injection of anti-\( hVPS34 \) antibody, and was reduced by 60–100% by injection of antisense oligonucleotides (data not shown).

We repeated the experiments in GRC-LR+73 cells, which are more easily acquisiced (Pollard and Stanners, 1979). Insulin stimulated DNA synthesis by more than 10-fold. Injection of AS1 and AS2 inhibited this insulin-stimulated DNA synthesis by 80 and 90%, respectively, as compared with a 70% inhibition achieved with anti-\( hVPS34 \) antibodies (Fig. 9A). AS3 was less effective, inhibiting DNA synthesis by ~50% (Fig. 9A). To test the
specificity of these effects, we synthesized scrambled versions of the more effective AS1 and AS2 oligonucleotides. These were only slightly inhibitory in comparison to the effects seen with the unscrambled oligonucleotides; AS1 and AS2 inhibited DNA synthesis by 87 and 91%, as opposed to 35 and 13% inhibition for their scrambled counterparts (Fig. 9B). These antisense experiments provide a confirmation of the antibody injection data, using entirely distinct reagents.

To better define the requirement for hVPS34 in the insulin-stimulated G1–S transition, we injected cells with anti-p110α or anti-hVPS34 antibodies before stimulating the cells or at various times after insulin stimulation. Consistent with Roche et al. (1994), we find that p110α is required throughout the first 6 h of insulin stimulation (Fig. 10). By 9 h of insulin stimulation, the cells become largely independent of p110α. Interestingly, the temporal requirement for hVPS34 was different than that for p110α. Although injection of anti-hVPS34 antibodies into cells after 3 h of insulin stimulation inhibited DNA synthesis by over 85%, injection at 6 h inhibited by only 50%, and injection at 9 h inhibited by only 20%. These data suggest that both hVPS34 and p110α are required for the insulin-stimulated G1–S transition. However, the requirement for hVPS34 is limited to an earlier period within G1 than the requirement for p110α.

Discussion

These data provide new insights into the role of different classes of PI 3′-kinase in distinct cellular events. We have examined a number of cellular responses that have been previously shown to be inhibited by low doses of the PI 3′-kinase inhibitor wortmannin. Our studies have focused on the wortmannin-sensitive class I (p110α) and class III (hVPS34) PI 3′-kinases. The class II PI 3′-kinase are significantly less sensitive to wortmannin and are unlikely to be involved in responses that are inhibited by 50–100 nM wortmannin (Vanhaesebroeck et al., 1997).

p110α can catalyze the production of PIP3, PIP2, and PIP3, whereas hVPS34 is limited to the production of PIP3 (Vanhaesebroeck et al., 1997). Previous studies have shown that cellular levels of PIP2 and PIP3 increase in mitogen-stimulated cells, whereas levels of PIP3 remain unchanged (Auger et al., 1989; Kapeller et al., 1991). Furthermore, the yeast VPS34 plays a role in the regulation of biosynthetic vacuolar sorting (Schu et al., 1993). Thus, our expectations before beginning these studies was that p110α would be involved in mitogen-stimulated responses such as rearrangement of the cytoskeleton and DNA synthesis, whereas hVPS34 would be involved in activities such as endocytic sorting. In fact, the situation is more complex. Both hVPS34 and p110α
are involved in vesicular trafficking and mitogenic signaling, albeit at different steps. In contrast, insulin-stimulated actin rearrangement requires p110α, but does not require hVPS34.

**PI 3'-Kinases and the Organization of the Actin Cytoskeleton**

Previous studies have shown that microinjection of Δp85 (a mutant p85 that lacks p110 binding sites) inhibits insulin-stimulated membrane ruffling in KB cells (Kotani et al., 1994). Furthermore, Martin et al. (1996b) have shown that microinjection of an activated p110α construct is sufficient to induce ruffling and stress fiber breakdown in 3T3-L1 adipocytes or rat-1 fibroblasts, whereas SH2 domains from p85 were inhibitory. Our finding that p110α is required for insulin-stimulated actin rearrangement is not surprising, but serves as a useful control for the specificity of the antibodies with regard to inhibition of p110α- and hVPS34-dependent responses in intact cells.

**PI 3'-Kinases in Early Endosomal Function**

Inhibition of hVPS34, but not p110α, has pronounced effects on two early endosomal events: the targeting of EE1 to the early endosome, and the postendocytic sorting of the PDGF receptor. These data suggest that the early endosome is a major site of hVPS34 action. Our finding that hVPS34 is responsible for the targeting of EE1 is consistent with several recent studies showing that the FYVE finger of EE1 binds specifically to the product of hVPS34, PI(3)P (Gaullier et al., 1998; Patki et al., 1998). Moreover, expression of EE1 in *S. cerevisiae* leads to a VPS34-dependent accumulation in intracellular membranes (Burd and Emr, 1998). EE1 in turn is required for homotypic fusion of early endosomes, and may function by recruitment of rab5 to the endosomal membrane (Mills et al., 1998; Simonsen et al., 1998). Thus, the hVPS34-dependent recruitment of EE1 plays a critical role in early endosomal function. It remains to be seen how hVPS34 itself is targeted to the endosomal mem-

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brane, perhaps via binding to the myristylated p150 (Panaretou et al., 1997). We also cannot yet explain the perinuclear localization of EEA1 in cells injected with anti-hVPS34 antibodies or treated with wortmannin. It is possible that this reflects the continued activity of a class II PI 3'-kinase, which would not be inhibited by either our antibodies or 100 nM wortmannin.

Inhibitory antibodies to hVPS34 also disrupt the postendocytic trafficking of the PDGF receptor, consistent with the ability of low-dose wortmannin to block PDGF receptor targeting and degradation (Joly et al., 1995). Although this could be due to an early endosomal defect, we cannot rule out additional sites of action at the late endosome or lysosome. The the involvement of hVPS34 in PDGF receptor trafficking may be analogous to the role of yeast VPS34 in vacuolar targeting (Herman et al., 1992), and is consistent with data from numerous laboratories showing that wortmannin inhibits both early and late endosomal fusion events and lysosomal delivery (for review see De Camilli et al., 1996).

Although our data suggest that p110α is not involved in early endosomal function, it should be noted that CHO cells contain both p110α and p110β. p110β also couples to p85 and should be similarly regulated by insulin or PDGF (Hu et al., 1993). Thus, it is possible that p85/p110β complexes play a role in the regulation of PDGF receptor sorting or EEA1 localization that augments the role of hVPS34. Such a requirement would be consistent with the finding that mutation of the p85 binding sites in the PDGF receptor mimic the effects of wortmannin on receptor sorting (Joly et al., 1994). The testing of anti-p110β antibodies is ongoing in the laboratory and will help to resolve this question.

**PI 3'-Kinases in Transferrin Recycling**

The recycling of transferrin receptors in Trvb-1 cells was significantly slowed by treatment of cells with wortmannin (Martys et al., 1996). The effects of this drug on the distribution of transferrin receptors was half-maximal at 30 nM wortmannin, consistent with the involvement of a mammalian class I or III PI 3'-kinase (Vanhaesebroeck et al., 1997). We now find that inhibition of both p110α and to a
lesser extent hVPS34 replicates the wortmannin phenotype with regard to transferrin recycling.

The role of the p85/p110α PI 3'-kinase as a positive regulatory of receptor recycling is consistent the fact that insulin, which activates p85/p110 (Ruderman et al., 1990), enhances the recycling of a number of constitutively recycling receptors (Wardzala et al., 1984; Davis et al., 1986). Moreover, the p85/p110 PI 3'-kinase is required for the insulin stimulation of Glut-4 recycling to the plasma membrane (Okada et al., 1994; Cheatham et al., 1994) and overexpression of p110α increases Glut 4 recycling in several systems (Martin et al., 1996a; Frevert and Kahn, 1997). Inhibition of hVPS34 also delays transferrin recycling, but to a much smaller extent. The different degrees of inhibition observed with anti-hVPS34 versus anti p110α antibodies could reflect differential recruitment of a regulatory protein that binds to PI[3,4,5]P3 with higher affinity than to PI[3]P. Alternatively, hVPS34 and p110α could act on distinct proteins within the recycling compartment.

PI 3'-Kinases in Mitogenic Signaling

The most striking result in this study is the requirement for the hVPS34 in insulin-stimulated DNA synthesis. This result has been verified using two entirely different approaches to reduce hVPS34 activity and/or expression. Our data suggest that hVPS34 is specifically required for entry of insulin-stimulated cells into S phase. Previous studies have shown that the levels of PI[3,4]P2 and PI[3,4,5]P3 increase acutely in response to mitogens such as insulin or PDGF, whereas the levels of PI[3]P does not change (Auger et al., 1989; Kapeller et al., 1991). hVPS34 is restricted to the production of PI[3]P, and it is therefore surprising that its activity is required for mitogenic signaling. It may be that constitutive levels of PI[3]P are required for cellular systems that are needed during the transition to S phase. Alternatively, PI[3]P levels may be acutely regulated in specific intracellular locations that are not detectable when whole cell lipid production is measured. Although we do not yet know the function of PI[3]P in mitogenic signaling, our data suggests that the products of hVPS34 are required only during the first 6 h of insulin stimulation. In contrast, the products of p110α are still critical at 6–9 h of insulin stimulation.

One can propose three general mechanisms by which hVPS34 could act during mitogenic signaling. First, as suggested above, the constitutive production of PI[3]P could be required for a cellular process that is needed during early G1. For example, it is possible that normal trafficking of tyrosine kinase receptors is required for efficient signal transduction. However, insulin receptor signaling is relatively normal in cells expressing a dominant-negative mutant of dynamin, which blocks coated pit-mediated endocytosis (Ceresa et al., 1998). Second, insulin could regulate the activity and/or subcellular distribution of hVPS34, leading to the production of PI[3]P at specific intracellular locales. This PI[3]P could in turn recruit specific PI[3]P-binding proteins involved in mitogenic signaling. Finally, a third hypothesis takes note of the potential conversion of PI[3]P to higher-order polyphosphoinositides. A recent finding by Anderson and colleagues (Zhang et al., 1997) shows that PI[4,5] kinases can convert PI[3]P to PI[3,4,5]P3.

If this pathway is a significant source of PI[3,4]P2 and PI[3,4,5]P3 in mitogen-stimulated cells, then reductions in the PI[3]P pool could affect intracellular levels of PI[3,4]P2 and PI[3,4,5]P3. In this way, changes in PI[3]P levels could influence the production of the 3-phosphoinositides that have known signaling functions (Toker and Cantley, 1997). Alternatively, two groups have identified a novel lipid, PI[3,5]P2, in yeast and mammalian cells (Dove et al., 1997; Tolias et al., 1998). In S. cerevisiae, PI[3,5]P2 is produced in response to osmotic stress by a pathway that requires VPS34 but is independent of the Hog1 mitogen-activated protein kinases (Dove et al., 1997). These data suggest a role for VPS34 in signal transduction, which would be consistent with our findings regarding hVPS34 and mitogenesis.

In summary, we have examined the roles of type I and type III PI 3'-kinases in mammalian cells. p110α and hVPS34 PI 3'-kinases both effect the endocytic system: hVPS34 regulates events in the early endosome and to a lesser extent the recycling compartment, whereas p110α primarily regulates the recycling pathway. Furthermore, both p110α and hVPS34 are necessary for insulin-stimulated DNA synthesis. However, the requirement for hVPS34 occurs during a narrower time window in G1 than the requirement for p110α, suggesting that these enzymes perform different functions in mitogenic signaling. The determination of the distinct roles played by different PI 3'-kinase isoforms adds a new layer of complexity to the functions of these lipid kinases in cellular signaling.

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