Phosphatidylinositol (PtdIns) 4-kinase catalyzes the synthesis of PtdIns-4-P, the precursor of an array of lipid second messengers generated by additional phosphorylation by PtdIns-4-P 5-kinase and PtdIns 3-kinase. PtdIns 4-kinase activity is conserved from yeast to higher eukaryotes. Multiple isoforms of mammalian PtdIns 4-kinase have been cloned and purified, and the activities have been detected in almost all subcellular locations. We previously reported the cloning and characterization of the first mammalian PtdIns 4-kinase named PI4Kα (Wong, K., and Cantley, L. C. (1994) J. Biol. Chem. 269, 28878–28884). Alternatively spliced forms of PI4Kα have also been identified from several sources including bovine brain (Gehrmann, T., Vereb, G., Schmidt, M., Klix, D., Meyer, H. E., Varsanyi, M., and Heilmeyer, L. M., Jr. (1996) Biochim. Biophys. Acta 1311, 53–63). Recently we isolated a distinct human PtdIns 4-kinase gene, named PI4Kβ, that encodes an enzyme that is wortmannin sensitive (Meyers, R., and Cantley, L. C. (1997) J. Biol. Chem. 272, 4384–4390). Here we report the locations of these enzymes and provide evidence for other yet unidentified isoforms present in specific organelles. PI4Kα is mostly membrane-bound and located at the endoplasmic reticulum; whereas PI4Kβ is in the cytosol and also present in the Golgi region. Neither of these isoforms accounts for the major type II PtdIns 4-kinase activity detected in the lysosomes and plasma membrane fraction.

The metabolism of phosphoinositides is a key event in transmitting mitogenic and developmental signals in response to a variety of hormones and growth factors. Two signal transduction pathways, each utilizing distinct phosphoinositide derivatives, have been characterized. In one pathway, phosphatidylinositol (PtdIns) 1,4,5-trisphosphate and diacylglycerol (4). PtdIns-4-P and PtdIns-4,5-P2 have also been shown to mediate actin rearrangement by directly regulating actin-binding proteins (5, 6). In a distinct pathway, phosphoinositides are phosphorylated at the C-3 position to generate a family of lipid messengers (7). These lipids, PtdIns-3-P, PtdIns-3,4-P2, and PtdIns-3,4,5-P3 are not substrates for phospholipase C but are implicated in mitogenesis (8), intracellular trafficking (9) as well as actin rearrangement (10, 11). Feeding into both PtdIns pathways is the precursor PtdIns-4-P, synthesized by PtdIns 4-kinases.

The subcellular distribution of mammalian PtdIns 4-kinases has been studied. The majority of PtdIns 4-kinase activity in human cells is membrane-bound (12). PtdIns 4-kinase activity is detected in most membrane structures, including plasma membrane (13), nuclear envelope (14), lysosome, Golgi apparatus (15), and endoplasmic reticulum (16). PtdIns 4-kinase activity is also detected in coated vesicles, glucose transporter-containing vesicles (17), and several specialized organelles, such as chromaffin granules (18) and secretory vesicles from mast cells (19). In addition, cytosolic PtdIns 4-kinases have been described (20). In view of such a wide subcellular distribution, it has long been speculated that different isoforms, targeted to different intracellular compartments, perform different physiological functions. While there is some evidence suggesting that distinct isoforms of PtdIns 4-kinase may be targeted to distinct organelles and be independently regulated, previous studies have been limited by lack of cDNA clones and isoform specific antibodies.

In Saccharomyces cerevisiae, only two PtdIns 4-kinase isoforms are found in the entire genome. PIK1, the first PtdIns 4-kinase to be cloned, encodes a nuclear-associated PtdIns 4-kinase of 125 kDa that is indispensable for cell growth (21, 22). Mutants arrest in G2 due to defects in cytokinesis. A second yeast gene, STT4, encodes a 200-kDa PtdIns 4-kinase that appears to be cytosolic and is dispensable for growth. Genetic studies of STT4 suggest its involvement in the protein kinase C pathway (23, 24). In contrast, the yeast PtdIns 3-kinase, Vps34, is required for vesicle trafficking from the Golgi apparatus to the vacuole (9). Interestingly FAB1, a yeast gene that is homologous to the mammalian PtdIns-4-P 5-kinase, is required for normal vacuole function and morphology (25). Because PtdIns 4-kinase generates PtdIns-4-P, the precursor for subsequent phosphorylation by downstream PtdIns kinases, PtdIns 4-kinases must play either a direct or an indirect role in vesicular trafficking (26).

Previously, we reported the cloning and characterization of the first mammalian PtdIns 4-kinase, named PI4Kα (1). This protein is highly homologous to the yeast STT4 enzyme. Northern blot analysis of the pol(A)+ mRNAs from human tissues and cell lines revealed multiple alternatively spliced transcripts. A 230-kDa PtdIns 4-kinase made from an alternatively spliced form of the rat PI4Kα has recently been described (27). A bovine gene encoding a 170–200-kDa PtdIns 4-kinase that shares more than 95% identity with the human PI4Kα in the overlapping region has also been reported (2). It represents probably another splice variant of PI4Kα. More recently, we isolated another human cDNA that encodes a 110-kDa PtdIns 4-kinase (named PI4Kβ) that is more homologous to the yeast PIK1 gene. PI4Kβ is wortmannin-sensitive and may be the
same enzyme recently reported to be involved in the hormone sensitive pools of inositol phospholipids (28). To determine the relative roles of these enzymes in producing phosphoinositides for membrane trafficking, hormone sensitive PtdIns turnover, growth factor-dependent PtdIns-3,4,5-P3 production, and cytoskeletal rearrangement, it is the first important to determine the subcellular locations of these enzymes.

**EXPERIMENTAL PROCEDURES**

*Materials—PtdIns, (γ,δ-F) ATP, and silica gel plates were purchased from Avanti (Alabaster, AL), DuPont NEN, and E. Merck (Germany), respectively. Mouse monoclonal antibody 12CA5, reactive to the influenza virus hemagglutinin, was purchased from BabCo. Rabbit polyclonal anti-Pi4Kα antibody 3334 was raised to a peptide corresponding to amino acids 501–512, KPYPKGDERKKA, coupled to keyhole limpet hemocyanin (1). Peptide antibodies were affinity-purified prior to immunoblotting and immunofluorescent staining experiments. Anti-Pi4Kβ antibody was raised against a GST-fusion protein that was generated by polymerase chain reaction using oligonucleotide primers that generate amino acids 410–538 of PI4Kα. The 2.6-kb cDNA fragment was tagged at the amino terminus with a 9-amino acid epitope (YPDPVDPYA) derived from influenza virus hemagglutinin by insertion into BlueScript encoding the epitope sequence. The tagged cDNA was subcloned into a mammalian expression vector pcDNA3 (Invitrogen). The construct was transiently transfected into CHO or HeLa cells using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer's procedures. The tagged cDNA was also subcloned into a baculoviral expression vector. Recombinant viruses were generated with the linear AcMNVP transfection module (Invitrogen) and were plaque-purified prior to use. The GST-Pi4Kβ construct was generated by polymerase chain reaction as described in detail elsewhere (3) and was expressed in *Escherichia coli* by standard procedures. The fusion protein is predicted to be 120 kDa since it lacks the amino-terminal 82 amino acids of PI4Kβ.*

**Preparation of Particulate and Cytosolic Fractions—** Adherent parental CHO-IRS or CHO-IRS cells transfected with PI4Kα were harvested with phosphate-buffered saline containing 1 mM EDTA and 1 mM EGTA. Cells were pelleted and then resuspended in homogenizing buffer (10 mM Tris-HCl, pH 7.4, 20 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin). After swelling for 15 min on ice, the cells were Dounce-homogenized (25–35 strokes). Nuclei and unbroken cells were spun out at 1000 × g for 10 min at 4 °C. The postnuclear supernatant was centrifuged at 100,000 × g for 1 h in a SW55Ti rotor (Beckman Instruments). The membrane pellet was solubilized with 1% Triton X-100 in homogenizing buffer. The cytosolic and particulate fractions were adjusted to equal volume.

**Separation of Subcellular Organelles—** Fractionation procedures were performed according to Storrie and Madden (29). Briefly, CHO-IRS cells transfected with PI4Kα were disrupted by low pressure nitrogen cavitation in 0.25 M sucrose, pH 7.4, containing various proteinase inhibitors. After centrifugation at 1300 × g for 10 min, the postnuclear supernatant was collected and overlaid on a hybrid percoll/metrizamide discontinuous density gradient (5 ml of 6% Percoll, 2 ml of 17% metrizamide, 2 ml of 35% metrizamide). The step gradient was centrifuged at 20,000 rpm for 30 min at 4 °C in a SW41Ti rotor (Beckman Instruments). Interfaces were sequentially removed from the top of the gradient as follows: first the top of the gradient, followed by the postnuclear supernatant in 0.25 M sucrose, the sucrose/Percoll interface, the 6% Percoll/17% metrizamide interface where lysosomes sediment, and finally the 17%/35% metrizamide interface where mitochondria sediment.

**Assays of Organelle Marker Enzymes—** Alkaline phosphodiesterase, cytochrome c oxidase, β-hexosaminidase, and α-mannosidase II were used as the marker enzymes for plasma membrane, mitochondria, lysosomes, and Golgi apparatus, respectively. Lactate dehydrogenase was used as a cytosol marker. Each of the enzyme assays was performed as described previously (29).

**Immunofluorescence Studies of PtdIns 4-Kinases—** HeLa cells grown on coverslips were fixed with 4% paraformaldehyde for 20 min. Fixed cells were permeabilized and nonspecific reactive sites were blocked for 30 min at room temperature in phosphate-buffered saline containing 0.1% Triton X-100, 5% normal goat and donkey serum. Cells were then incubated with the appropriate primary PtdIns 4-kinase isofrom-specific antibody for 1 h at room temperature. Primary antibodies were detected by species-specific secondary antibodies, namely C9T7-conjugated goat anti-rabbit IgG and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG. ER was identified by staining BiP, a resident ER protein. Golgi was visualized using anti-β-adaptin. The immuno- stained cells were observed by either conventional light or confocal microscopy.

**PtdIns Kinase Assay and HPLC Analysis—** PtdIns kinase assays were performed as described previously (30). Briefly, the reaction mixture contained 0.3% Triton X-100, 50 μM ATP, 20 μM HEPES, pH 7.5, 10 mM MgCl2, 0.2 mg/ml sonicated lipids, 20 μCi of (γ-32P)ATP (3000 Ci/mmol; DuPont NEN) per sample. Assays were performed at 37 °C for 20 min and then stopped with 25 μl of 5 N hydrochloric acid. The lipid was extracted with 160 μl of 1:1 (v/v) chloroform:methanol. The organic layer was collected and analyzed by both thin layer chromatography and HPLC as described in detail elsewhere (31).

**RESULTS**

**PtdIns 4-Kinase Antibody Specificity—** Since the 97-kDa form of PI4Kα and the PI4Kβ enzyme have similar mobilities on SDS-gels, it is critical to verify that the antibodies raised against the highly divergent regions of these two enzymes indeed do not cross-react. We assessed the isozyme specificity of the PtdIns 4-kinase antibodies by immunoblotting the recombinant PI4Kα and PI4Kβ that were expressed in Sf9 insect cells and in *E. coli*, respectively. Fig. 1 shows that PI4Kα antibody detected a single band of 97-kDa protein only in Sf9 expressing PI4Kα (lane 2) and lysates prepared from S9 cells expressing human PI4Kα (lane 3) or wild-type viral proteins (lane 4) were immunoblotted first with anti-PI4Kα antibodies (left panel), and then re probed with anti-PI4Kβ antibodies (right panel).
genes are almost ubiquitously expressed in human tissues (1, 3), and since the antibodies raised against these enzymes react with the respective proteins in other mammalian tissues (not shown), we first investigated the ability of these antibodies to detect proteins in CHO cells. The anti-PI4Ka antibodies did not detect a 97-kDa PI4Ka isoform in the parental CHO cells (Fig. 2A, left panel), but strongly reacted with an approximately 180-kDa protein (p180) of the size expected for the high molecular weight alternative splice of PI4Ka (2). This result is consistent with the ubiquitous expression of the 7.5-kilobase pair message for PI4Ka (1, 27). The 97-kDa form of PI4Ka was detected when this cDNA was introduced. The PI4Kβ antibody reacted with an approximately 110-kDa band (Fig. 2A, right panel), consistent with the size of this protein previously detected in Jurkat cells (3). The postnuclear supernatants from the PI4Ka-transfected cells were separated into soluble and particulate fractions by centrifugation at 100,000 × g. The 97-kDa PI4Ka was predominantly associated with the particulate fraction (Fig. 2B, left panel), as was p180. p110 PI4Kβ was found predominantly in the soluble fraction (Fig. 2B, right panel).

To further investigate the intracellular localization of PtdIns 4-kinase isoforms, cells overexpressing PI4Ka were fractionated using the hybrid Percoll/metrizamide discontinuous density gradient as described previously (29). This gradient allows the isolation of lysosomes, mitochondria, and partial separation of plasma membrane from cytosol and organelles such as ER and Golgi. Interfaces sequentially collected from the top of the step gradient were adjusted to the same volume. Equal portions of individual fractions were then used for organelle marker enzyme assays, and for Western blot analysis with the anti-PtdIns 4-kinase antibodies. Monitoring the β-hexosaminidase activities, we found 85% of the lysosomes sedimented at the interface of Percoll/17% metrizamide as expected (Table I). The cytochrome c oxidase assays indicated that 69% of the mitochondria sedimented to the 17%/35% metrizamide interface, and 22% remained in the Percoll/17% metrizamide interface. Western blot revealed no significant amounts of anti-PI4Ka or anti-PI4Kβ reactive protein present in these two fractions. Instead, p97 PI4Ka, PI4Kβ, and the anti-PI4Ka reactive p180 concentrated at the top of the gradient and in the 0.25 to sucrose fraction (Fig. 3), where over 95% of Golgi apparatus and 40% of plasma membrane cofractionated (Table I). About 40% of the plasma membrane marker activity was also present at the sucrose/Percoll interface, but PI4Ka and β were not detected in this fraction, suggesting that they are not associated with this fraction of the plasma membrane.

The various subcellular fractions were also assayed for total PtdIns 4-kinase activity. Although half of the PtdIns 4-kinase activity was detected in the upper two fractions where cytosol, Golgi apparatus, and endoplasmic reticulum partition, and where both PI4Ka and PI4Kβ fractionate (Fig. 3), a substantial amount of activity was detected in the region where plasma membranes sediment (34%) and where lysosomes sediment (15%) (Table I). The assay was carried out under conditions in which PtdIns 3-kinase is inactive (0.3% Triton X-100). HPLC analysis of the deacylated products generated under these conditions verified the absence of PtdIns-3-P (Fig. 4). The PtdIns 4-kinase activity was not significantly inhibited by 1 μM wortmannin, indicating that the wortmannin-inhibitable PI4Kβ is not a major component of total activity in these fractions (data not shown). Consistent with previous studies, an inhibitory antibody, 4C5G, raised against the 55-kDa type II PtdIns 4-kinase caused about 75% inhibition of the plasma membrane PtdIns 4-kinase (sucrose/Percoll fraction, Table I). This antibody also substantially inhibited PtdIns 4-kinase activity in other fractions. The 55-kDa type II PtdIns 4-kinase was previously shown to be the major PtdIns 4-kinase in red cell plasma membrane (32) and in other cells. Thus, the results in Table I indicate that the membrane bound forms of PI4Ka and PI4Kβ are in compartments of the cell consistent with Golgi apparatus and/or endoplasmic reticulum, and that the major PtdIns 4-kinase activity in plasma membrane and lysosome is due to a type II PtdIns 4-kinase distinct from the PI4Ka isoforms and from PI4Kβ.

Immunofluorescent Localization of PtdIns 4-Kinase Isoforms—To further discern the intracellular localization of PI4Ka and β, cytoimmunofluorescence studies were performed using both conventional and confocal microscopy. We chose
Fractons of subcellular organelles obtained from the hybrid Percoll/metrizamide gradient (see “Experimental Procedures”) were assayed for organelle-specific enzyme activities. The percentage of total activity of each organelle marker is presented. Fractions from the same experiment were also assayed for lipid kinase activity. The PtdIns 4-kinase isozymes are targeted to distinct subcellular compartments and provide support for the hypothesis that individual members of this family of PtdIns 4-kinases are targeted to distinct subcellular compartments and hence are performing different cellular functions. PI4Kα is detected predominantly in the particulate fraction, and PI4Kβ is present in the cytosol and to a lesser extent in the particulate fraction. Fractionation of CHO-IRS cells revealed that neither PI4Kα nor PI4Kβ is present in the cytosol, while PI4Kβ is present in the particulate fraction (solid line). The distribution of PI4K isozymes is indicated on the HPLC analysis of PtdIns-P. The standards [γ-32P]PtdIns-4-P and [γ-3H]PtdIns-4,5-P2 are shown (dotted line), and the migration position of [γ-3H]PtdIns-3-P is indicated. Presented here is the profile of the PtdIns-P product generated from the top fraction of the gradient (solid line). PtdIns-P products from other fractions also co-migrate exclusively with the PtdIns-4-P standard (not shown).

**FIG. 4.** HPLC analysis of PtdIns-P. HPLC analysis was performed on the PtdIns-P products generated from the lipid kinase assay as described under “Experimental Procedures.” The standards [γ-3H]PtdIns-4-P and [γ-3H]PtdIns-4,5-P2 are shown (dotted line), and the migration position of [γ-3H]PtdIns-3-P is indicated. Presented here is the profile of the PtdIns-P product generated from the top fraction of the gradient (solid line). PtdIns-P products from other fractions also co-migrate exclusively with the PtdIns-4-P standard (not shown).
the α nor β isoform is located in the lysosomes or mitochondria, and probably not in the plasma membrane. Immunocytofluorescence studies demonstrate that PI4Ka is present in the endoplasmic reticulum, and PI4Kb is localized to the Golgi region.

While this work was in progress, Nakagawa et al. (27) reported the cloning and characterization of an alternative splice of rat PI4Ka gene that generates a protein predicted to be 230 kDa. Gehrmann et al. (2) also isolated a partial bovine cDNA encoding a 170–200-kDa PtdIns 4-kinase, that is almost certainly another splice variant of PI4Ka. The carboxyl-terminal half of both high molecular weight proteins contains the domains necessary for lipid catalysis, and share greater than 95% identity with the human p97 PI4Ka. We also noted an alternative splice of PI4Ka from human tissues that is predicted to encode a larger protein (1). Nakagawa et al. (27) found that the 97-kDa PI4Ka and the higher molecular weight form colocalize, and the later is predominantly associated with the particulate fraction. These results are consistent with our finding that the p180 and p97 cofractionate and colocalize. It is unlikely that they are integral membrane proteins, since neither splice variant contains a predicted transmembrane domain. However, it is clear from our studies that the membrane association does not require the extended amino-terminal sequence of the higher molecular weight form of PI4Ka.

Nakagawa et al. (27) reported that Flag-tagged versions of both forms of PI4Ka localize to the Golgi membrane in COS cells. In our study, both the exogenously expressed p97 PI4Ka and the endogenous p180 localize to the ER of HeLa cells. The discrepancy cannot be explained by the cell type, since we also obtained ER staining when PI4Ka was expressed in COS cells (not shown). The possibility that the HA-tag affected localization is unlikely, since the endogenous p180 exhibited the same location as the HA-tagged p97.

By separating intracellular structures on a step gradient and
assaying each fraction for PtdIns kinase activity, we have shown that the lysosomal and plasma membrane-associated PtdIns 4-kinase activities are unlikely to be the result of PI4Kβ or any splice variants of PI4Kα. A plausible candidate is the previously described 55-kDa type II PtdIns 4-kinase. Historically, PtdIns 4-kinases are classified into two categories, types II and type III, on the basis of their molecular sizes, and the differences in their sensitivity to adenosine and nonionic detergent (13). The type II PtdIns 4-kinase is dramatically activated by nonionic detergent but is potently inhibited by both adenosine and the 4C5G monoclonal antibody (32). Type II PtdIns 4-kinase has been purified from erythrocyte membrane (32) and also copurified with the epidermal growth factor receptor (35), as well as with the lymphocyte CD4 antigen (36). Additional findings have also demonstrated that it is recruited for its ability to function in association. Future studies with dominant negative forms or any splice variants of PI4Kα are unlikely to be the result of PI4Kβ since they share similar enzymatic properties. However, we are unable to detect proteins of 55 kDa resulting from the processing of the 97-kDa PI4Kα. A significant amount of PI4Kα gene knockouts will elucidate the roles of these enzymes in membrane traffic. Our results do not exclude other possibilities that the type II 55-kDa enzyme is a proteolytic product of the 97-kDa PI4Kα since they share similar enzymatic properties. However, we are unable to detect proteins of 55 kDa resulting from the processing of the 97-kDa PI4Kα. Thus, the 55-kDa type II PtdIns 4-kinase appears to be encoded by a distinct but related gene.

The finding of the differential ER and Golgi localization of PI4Kα and PI4Kβ suggests a direct role of PI4Kα and β in ER and Golgi function, as opposed to the ligand-stimulated phosphoinositide turnover at the plasma membrane. The finding that PI4Kβ is Wortmannin-sensitive (IC₅₀, 140 nM) and is located in the Golgi apparatus should caution future studies employing wortmannin inhibition as the standard of implicating the role of PtdIns 3-kinase in targeting proteins from Golgi apparatus to lysosome. Finally, our results do not exclude other sites of PtdIns 4-kinase function. For example, the colocalization of ER marker was not complete for PI4Kα. We also noticed a significant amount of PI4Kα in the particulate fraction that could not be extracted with detergent, implicating cytoskeletal association. Future studies with dominant negative forms or gene knockouts will elucidate the roles of these enzymes in mammalian cell function.

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