We report herein the cloning and characterization of a novel class II phosphoinositide 3-kinase, termed P13K-IIγ, from the cDNA library of regenerating rat liver. This cDNA encodes a protein of 1505 amino acids with a calculated molecular mass of 170,972 Da. The amino acid sequence of P13K-IIγ is highly similar to that of class II PI 3-kinases, including murine Cpk-m/p170 and human HsC2-PI3K. It contains a C2 domain at its C terminus but no recognizable protein motifs at its N terminus. P13K-IIγ displays a restricted substrate specificity for PtdIns and PtdIns 4-P, but not for PtdIns 4,5-P2. By epitope tag immunocytochemistry, the immunoreactivity for P13K-IIγ is localized in the juxtanuclear Golgi region at high levels and also in the plasma and nuclear membranes at low levels. By Northern blot analysis and in situ hybridization histochemistry, P13K-IIγ mRNA expression is confined to the liver throughout the development with much higher expression in adult liver than in fetal liver. In addition, its expression increases during liver regeneration after partial hepatectomy with maximal expression after the growth period, suggesting that P13K-IIγ may function mainly in highly differentiated hepatic cells.

Phosphoinositide 3-kinase (PI 3-kinase)1 catalyzes the phosphorylation of phosphoinositides such as phosphatidylinositol (PtdIns), PtdIns 4-P, and PtdIns 4,5-P2 at the D3 position of the inositol ring to generate PtdIns 3-P, PtdIns 3,4-P2, and PtdIns 3,4,5-P3, respectively. Although the intracellular level of PtdIns and PtdIns 4-P, but not for PtdIns 4,5-P2. By epitope tag immunocytochemistry, the immunoreactivity for P13K-IIγ is localized in the juxtanuclear Golgi region at high levels and also in the plasma and nuclear membranes at low levels. By Northern blot analysis and in situ hybridization histochemistry, P13K-IIγ mRNA expression is confined to the liver throughout the development with much higher expression in adult liver than in fetal liver. In addition, its expression increases during liver regeneration after partial hepatectomy with maximal expression after the growth period, suggesting that P13K-IIγ may function mainly in highly differentiated hepatic cells.

EXPERIMENTAL PROCEDURES

Reverse Transcription-Polymerase Chain Reaction—Regenerating liver was prepared from partially hepatectomized rats 24 h after the removal of two-thirds of liver mass according to the method of Higgins.
and Anderson (20). Total RNA was prepared by the guanidine method, and poly(A) RNA was isolated by chromatography on an oligo(dT)-cellulose column. First strand cDNA was prepared using First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech). Oligonucleotide primers corresponding to two regions of highly conserved amino acid sequences in PI kinase domains, (V/T)GDD(C/L)RQ (5-end) and (G/A)TG-3-end (underlined) were used for PCR amplification as sense and antisense primers. The 5'- ends of both primers contained an EcoRI restriction site (Amersham Pharmacia Biotech). At least five overlapping RACE products were subcloned and sequenced. 

**cDNA Cloning—Oligo(dT)-primed cDNA library from the regenerating liver of 49-day-old (P49) rat was constructed in the same way as described previously (21). Clones (approximately 2×10^6) derived from the cDNA library were screened by hybridization with the 400-base pair PCR products labeled with [32P]dCTP. A positive bacteriophage containing a 1.4-kb insert was isolated. The insert (pFSK4) was cloned into pBluescript, and the sequence was determined by the dideoxy chain termination method with a 377 DNA sequencer (Applied Biosystems). The missing 5' end was obtained by a rapid amplification of cDNA ends (RACE) using two primers corresponding to two regions of highly conserved amino acid sequences in PI kinase domains, (V/T)GDD(C/L)RQ (5-end) and (G/A)TG-3-end (underlined). PCR amplification was performed using AmpliTaq DNA polymerase as follows: 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min for 30 cycles. The reaction products of approximately 400 base pairs were then subcloned and sequenced.

**Expression and Preparation of a Novel Molecule and Assay for PI 3-Kinase Activity—A NotI site was introduced by PCR at the position of the initiation codon of a newly identified cDNA using the primer (5'-GGCGCGCCGCAAATATGGCATACATGGC-3'). Resulting NotI fragments were ligated to the same site of the expression vector, pSH (peDL-Sro296 in Ref. 22) as modified by Sakane et al. (23). At the same time, FLAG marker peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Lys (Kodak Co.), was fused upstream of the initiation codon, ATG, of the cDNA for the newly identified molecule. FLAG-tagged molecule was recovered as described below from COS-7 cells 3 days after the transfection by the DEAE-dextran method (24). Cells were harvested and lysed in lysis buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1% dithiothreitol, 1% diethylamine, 1% (dT)18, 0.1% Nonidet P-40, or CHAPS) were added to the reaction buffer. Alternatively, 3.5 mg/ml aprotinin, 200 mg/ml soybean trypsin inhibitor). The lysate was clarified by centrifugation (16,000 × g, 15 min), and its protein concentration was determined by the method of Lowry et al. (25). The supernatant was incubated in the presence of 40 μg/ml soybean trypsin inhibitor). The lysate was clarified by centrifugation (16,000 × g, 15 min), and its protein concentration was determined by the method of Lowry et al. (25). The supernatant was incubated with anti-FLAG antibody-agarose beads (approximately 20 μl of beads/1 mg of lysate) for 90 min. The resulting beads were washed repeatedly and resuspended in assay buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 3.5 mM MgCl2). A volume of 10 μl of the beads recovered from 100 μg of the transfected cell lysates was then used for PI kinase assay. After 10 min of incubation with lipid substrates (200 μM each of PtdIns, PtdIns 4-P, and PtdIns 4,5-P2), the reaction was carried out for an additional 10 min in the presence of 40 μM MgATP. Various concentrations of wortmannin or detergents (Triton X-100, Nonidet P-40, or CHAPS) were added to the reaction buffer. Alternatively, 3.5 mM Mn2+ was added instead of Mg2+. Reaction products were extracted and resolved by thin layer chromatography (TLC) using Silica Gel 60 plates (Whatman). For determination of the phosphorylated position on the lipid substrates, the reaction products were separated by TLC using a borate buffer system as described by Walsh et al. (26). As reference, [γ-32P]ATP and [γ-32P]ATP. Various concentrations of wortmannin or detergents (Triton X-100, Nonidet P-40, or CHAPS) were added to the reaction buffer. Alternatively, 3.5 mM Mn2+ was added instead of Mg2+. Reaction products were extracted and resolved by thin layer chromatography (TLC) using Silica Gel 60 plates (Whatman). For determination of the phosphorylated position on the lipid substrates, the reaction products were separated by TLC using a borate buffer system as described by Walsh et al. (26).
PtdIns 3-P, PtdIns 3,4-P 2, and PtdIns 3,4,5-P 3, the products were re-
solved by TLC in a buffer consisting of chloroform/acetone/methanol/
acetic acid/water (80/30/26/24/14).

RNA Extraction and Northern Blot Analysis—Poly(A) 1 RNAs were
purified from liver, testis, brain, heart, lung, and spleen of the P49 rat,
livers of prenatal day 18 (E18) rat, and from regenerating livers of P49
rats on the 1st, 2nd, and 3rd day after partial hepatectomy (PH1, PH2,
and PH3, respectively) as described above. Each of the poly(A) 1 RNA
samples (2 or 5 \( \mu \)g) was denatured with formamide and size-separated
by agarose gel electrophoresis. The RNAs were transferred and fixed to
a nylon membrane (Nytran, Schleicher & Schuell) and hybridized with
a [32P]dCTP-labeled probe prepared from a 6-kb Not I restriction frag-
ment of a novel cDNA. Conditions for hybridization and washing were
performed as described previously (21). The intensity of each band was
measured by a Bio-Image Analyzer (Fuji Co.).

In Situ Hybridization Histochemistry—Fresh frozen blocks of livers
and brains from adult (P49) and 7-day-old (P7) rats, whole bodies of
fetal (E15 and E18) rats, and regenerating livers of P49 rats after
partial hepatectomy (PH1, PH2, and PH3) were sectioned at 30
\( \mu \)m thickness on a cryostat. The sections were hybridized for 16 h at 42 °C
with [35S]dATP-labeled oligonucleotides (5'-GGCTCACTCTGTAGTGT-
CATGCTGAGAAGCCTAGACCCCAGCGGA-3' (nucleotides 1944–
1988)). After hybridization, the sections were rinsed twice in 2\( \times \)
SSC, 0.1% Sarkosyl at 42 °C for 20 min, three times in 0.1
\( \times \) SSC, 0.1%
Sarkosyl at 42 °C for 1 h, and dehydrated in 70 and 100% ethanol. For
comparison in relative hybridization intensity, targeted tissue sections
were all mounted on one glass slide and exposed to a Hyperfilm-
max (Amersham Pharmacia Biotech) for 3 weeks.

Immunocytochemistry and Immunoblotting—The tissues and cells
were fixed with 4% paraformaldehyde, 0.01% Triton X-100 and were
incubated with the anti-FLAG antibodies (Anti-FLAG M2, Kodak).
Sites of antigen-antibody reaction were visualized using the avidin-
biotin complex system (Vector Laboratories) with diaminobenidine as
a substrate. In the immunoblotting, the lysates of overexpressed cells
were collected by centrifugation (550 × g, 10 min), and the supernatant
was further centrifuged at 100,000 × g for 30 min to separate soluble
and particulate fractions. The proteins of both fractions were subjected
to SDS-7.5% polyacrylamide gel electrophoresis and then transferred
to a polyvinylidine difluoride membrane. The membrane was incubated
for 2 h at room temperature with anti-FLAG antibodies and treated
with peroxidase-conjugated anti-mouse IgG antibodies for 1 h.

RESULTS

The deduced amino acid sequences of the composite cDNA of
the novel molecule are presented in Fig. 1. The putative initia-
tion codon was preceded by in-frame stop codon at nucleotide
–57. The predicted open reading frame encoded a protein of
1505 amino acids with a calculated molecular mass of 170,972
Da. When the sequences of the extreme 3\( ' \) and 5\( ' \) ends of the
composite cDNA were used in the PCR amplification, a full-
length cDNA of the same size was amplified, and its sequence
was identical to that of the composite cDNA. The deduced
amino acid sequence of the novel molecule contained a lipid
kinase unique domain, a putative catalytic domain, and a C2
domain as found previously for murine Cpk-m/p170 and human
HsC2-PI3K (9–11). The novel molecule showed 48, 42, and 30%
identities to HsC2-PI3K, Cpk-m/p170, and p110\( \alpha \) in the lipid
kinase unique domain and 59, 57, and 42% identities to the
three individual kinases in the catalytic domain, respectively.
The C2 domain of the novel molecule was located at the C
terminus, and it was 40 and 32% identical to the same domain
of HsC2-PI3K and Cpk-m/p170, respectively. The N terminus
of the novel molecule did not contain any recognizable protein
motifs (Fig. 2).

In Northern blot analysis of several tissues of P49 rat, a
distinct hybridization band of 7 kb was detected only in the
liver. A smaller transcript of approximately 2.5 kb was detected
at a low level in the heart and testis, which was considered to
be a result of alternative splicing. No significant hybridization
Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

The expression of GAPDH was detectable, though weakly, in E18 liver, and the expression level of P49 liver was approximately 10-fold higher than that of E18 liver (Fig. 3A). During liver regeneration after partial hepatectomy, the mRNA expression showed a pattern of time-dependent gradual increase, and it increased markedly on the 3rd day after hepatectomy (PH3) (Fig. 3C). The expression level of PH3 was superficially 3-fold higher and 1.6-fold higher than that of PH0, even after normalizing it to GAPDH mRNA expression, although a previous study has shown a slight increase in PH1, even after normalizing it to GAPDH mRNA expression, compared with immunoprecipitates from lysates of cells transfected with cDNA for PI3K-IIγ, whereas no formation of [32P]PtdIns 3,4,5-P3 was discerned (29), which was also confirmed by us.

By in situ hybridization histochemistry of the whole body of embryos on E15 and E18 and of liver and brain on P7 and P49, the expression signals for this novel molecule were detected only in the liver, and no significant signals were detected in any other tissues of embryos and the postnatal brain (Fig. 4).

Detection of the lipid kinase activity of the novel molecule was performed using immunoprecipitates from lysates of COS-7 cells transfected with the corresponding epitope-tagged cDNA. A single band at an approximate size of 190 kDa was visualized by silver staining on SDS-polyacrylamide gel (Fig. 5A). A single immunoreactive band of the same size was detected at almost equal intensities in both soluble and particulate fractions (Fig. 5B). In an assay for lipid kinase activity using PtdIns as a substrate, the selective production of [32P]PtdIns 3-P, but not [32P]PtdIns 4-P, was clearly revealed, compared with immunoprecipitates from lysates of cells transfected with cDNAs for 92-kDa PtdIns 4-kinase (28) as well as lysates of A431 cells, which are known to have high intrinsic activities for both PtdIns 3-kinase and PtdIns 4-kinase (27) (Fig. 5C). When PtdIns 4-P and PtdIns 4,5-P2 were used as substrates, the formation of [32P]PtdIns 3,4-P2 was detected, whereas no formation of [32P]PtdIns 3,4,5-P3 was discerned (Fig. 5D). The kinase activity was inhibited by wortmannin.
with an IC$_{50}$ of 12 nM, whereas the PI 3-kinase activity of A431 cell lysates was inhibited with an IC$_{50}$ of 5 nM under the same conditions. The kinase activity was also inhibited almost completely by addition of 0.5% Triton X-100, Nonidet P-40 or CHAPS, or in the presence of 3.5 mM Mn$^{2+}$ instead of Mg$^{2+}$ (data not shown).

When COS-7 cells were transfected with the epitope (FLAG)-tagged cDNA for the novel molecule and immunostained for the FLAG-tag, immunoreactive cells accounted for about 20% of the total cell populations and appeared randomly dispersed in each culture dish. The immunoreactive products were densely aggregated in juxtaposition to the cell nuclei. In addition, the contours of the cell nuclei and cell boundary appeared clearly delineated (Fig. 6). When the transfection was made with a cDNA without the tag, no immunoreactivity was detected (data not shown).

**DISCUSSION**

Judging from the presence of a C2 domain in its molecular structure as well as its substrate specificity for PtdIns and PtdIns 4-P in vitro and inhibition of its kinase activity by wortmannin and Mn$^{2+}$, it is clear that this novel molecule represents the third species of class II PI 3-kinase in vertebrates. But it is the first vertebrate species whose biochemical characteristics and localization in tissues and cells have been revealed in detail (9–11). The novel molecule is thus termed as Liver-specific Class-II PI3K of Rat and for proteins that may modulate the action of this molecule.

The appearance of PI3K-IIy in both supernatant and particulate fractions, together with the absence of any transmembrane domains in its deduced amino acid sequence, suggests that translocation of PI3K-IIy occurs between the two intracellular components although the molecular mechanism of the translocation remains to be determined. The present immunohistochemical analysis of COS-7 cells overexpressed with PI3K-IIy shows that the immunoreactivity for PI3K-IIy is localized in the juxtanuclear Golgi region with the highest intensity and, furthermore, in the nuclear and plasma membranes at low levels. Whether or not the localization of the immunoreactivity in the juxtanuclear Golgi region by light microscopy represents the localization of this molecule in the Golgi apparatus or in both the Golgi and adjacent endoplasmic reticulum requires further double immunocytochemical analysis using specific antibodies against the two organelles. It will also be necessary to use a specific antibody against PI3K-IIy to examine whether the present immunohistochemical finding represents the real localization or reflects the artificially high production of PI3K-IIy protein in such cells.

The present study reveals that the gene expression of PI3K-IIy is confined to the liver at both prenatal and postnatal stages, with the latter at a much higher level. In addition, its expression increases during liver regeneration after partial hepatectomy in a time-dependent manner. Haber et. al. has recently defined three chronological patterns of expression for various genes in the regenerating liver after hepatectomy. The first pattern parallels the major growth period of the liver that ends at 60–72 h after hepatectomy. The second has two peaks coincident with the first and second G1 phases of the two hepatic cell cycles. In the third pattern, the expression level reaches the maximum at 72 h, which is maintained for a substantial length thereafter (31). The induction pattern of PI3K-IIy mRNA after partial hepatectomy is similar to the third pattern. These results may suggest that PI3K-IIy is not involved in the major mitogenic signaling pathway but is involved in other pathways responsible for some yet undefined liver-specific matured functions, in contrast to the expectation mentioned in the introduction. C/EBPα (CCAAT/enhancer binding protein α) represents one of the molecules exhibiting the third pattern of the gene expression in the regenerating liver and is expressed late in gestation and highly in the non-growing normal adult liver (32, 33). C/EBPα has also been shown to activate the transcription of several tissue-specific genes such as insulin-responsive glucose transporter 4 (GLUT4) and phosphoenolpyruvate carboxykinase (PEPCK) in a coordinated fashion (34, 35). The functional relation of PI3K-IIy to C/EBPα in the liver remains to be evaluated. With the regard to the possible functional significance of PI3K-IIy in matured cells, it should be noted that the axonal crush was shown in our recent study to induce the enhanced gene expression of a class I PI 3-kinase in hypoglossal motoneurones which represent the non-mitotic cell population (36). We have also shown in crushed/axonotomized motoneurones the enhanced expression of a serine/threonine protein kinase, Akt/PKB, which is activated by PtdIns 3,4,5-P$_3$ but not by PtdIns 3,4,5-P$_3$. PtdIns 3,4-P$_2$ is a direct product of the class II PI 3-kinase from PtdIns 4-P although it is also a product of the hydrolysis at the d-5 position of PtdIns 3,4,5-P$_3$ which is produced by class-I PI 3-kinase (37). To understand the physiological role of PI3K-IIy in non-mitotic signaling in hepatic cells, further studies will be needed such as a search for agonists that may induce enhanced activation of PI3K-IIy and for proteins that may modulate the action of this molecule.

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