Cloning of cDNAs Encoding Two Isoforms of 68-kDa Type I Phosphatidylinositol-4-phosphate 5-Kinase*

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Accumulating evidence suggests that phosphatidylinositol metabolism is essential for membrane traffic in the cell. Of particular importance, phosphatidylinositol transfer protein and the type I phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) have been identified as cytosolic components required for ATP-dependent, Ca\textsuperscript{2+}-activated secretion. In order to identify PI4P5K isoforms that may play important roles in regulated insulin secretion from pancreatic β-cells, we employed the polymerase chain reaction with degenerate primers and screening of a cDNA library of the murine pancreatic β-cell line MIN6. Two novel cDNAs, designated PI4P5K-I\textalpha and PI4P5K-I\textbeta, were identified, which contained complete coding sequences encoding 539- or 546-amino acid proteins, respectively. These cDNAs were expressed in mammalian cells with an adenoviral expression vector. Proteins of both isoforms migrated at 68 kDa on SDS-polyacrylamide gel electrophoresis and exhibited phosphatidylinositol-4-phosphate 5-kinase activity, which was activated by phosphatidic acid, indicating that these proteins were type I isoforms. While the amino-terminal regions differ significantly, Northern blot analysis depicted that tissue distributions differed between the two isoforms. Molecular identification of type I PI4P5K isoforms in insulin-secreting cells should provide insights into the role of phosphatidylinositol metabolism in regulated exocytosis of insulin-containing large dense core vesicles.

Exocytotic release of neurotransmitters and hormones is a highly complex process. In addition to Ca\textsuperscript{2+} and ATP, regulated fusion of secretory granules with the plasma membrane requires membrane proteins, v-SNARE\textsuperscript{1} and t-SNARE. Also required are cytosolic proteins, including N-ethylmaleimide-sensitive factor and soluble N-ethylmaleimide-sensitive factor attachment proteins (reviewed in Ref. 1). Recently, two other cytosolic protein components required for ATP-dependent priming for Ca\textsuperscript{2+} -activated secretion have been identified: phosphatidylinositol transfer protein (2) and the type I phosphatidylinositol-4-phosphate 5-kinase (PI4P5K) (3). PI4P5K produces, from phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P\textsubscript{2}), which has been demonstrated to be important in various cellular processes. PtdIns(4,5)P\textsubscript{2} acts as a substrate for phospholipase C, generating the major second messenger molecules, inositol 1,4,5-trisphosphate and diacylglycerol (4). In addition, PtdIns(4,5)P\textsubscript{2} itself has also been demonstrated to function as a regulator molecule in several cellular processes, including actin filament reorganization (5, 6) and exocytosis (7). More recently, it has been postulated that PtdIns(4,5)P\textsubscript{2} functions in the fusion of intracellular vesicles with target membranes (8). Despite these important roles played by the type I PI4P5K, molecular identification of the enzyme has yet to be carried out.

Insulin secretion from pancreatic β-cells is one example of regulated exocytosis. Extensive studies have been conducted, which suggest the importance of the polyphosphoinositide synthesis pathway and the breakdown of its products by phospholipase C (9–11). We recently demonstrated that insulin secretion requires an ATP-dependent process (12), suggesting that the type I PI4P5K plays a significant role in insulin secretion from pancreatic β-cells. We have therefore made efforts to identify PI4P5K-like proteins in insulin-secreting MIN6 cells (13). Here, we have identified two type I isoforms of PI4P5K from a cDNA library of the murine pancreatic β-cell line MIN6.

EXPERIMENTAL PROCEDURES

Screening of MIN6 Cell cDNA Library—Two blocks of six amino acids, (Y/F/D/LKGS and MISTRYLV), were chosen for the synthesis of degenerate oligonucleotide primers. These two sequences are highly conserved among human type II or type C PI4P5K (14, 15) and its yeast homologues, Mss4p (16) and Pahlp (17). The sequences of the sense primers were 5′-T(A/T)(C/T)GA(C/T)AA(A/G)GG(A/C/G/A)GT(AG-3′)(S1), 5′-T(A/T)(C/T)GA(A/G)AG(A/G)CT(A/C/G/T)TAA(A/G)GG(A/C/G/A)GT(AG-3′)(S2), 5′-T(A/T)(C/T)GA(C/T)TT(A/G)AA(A/G)GG(A/C/G/A)GT(AG-3′)(S3) and 5′-T(A/T)(C/T)GA(C/T)TT(A/G)AA(A/G)GG(A/C/G/A)GT(AG-3′)(S4), and those of the antisense primers were 5′-A(A/C/G)(C/T)AA(A/G)GG(A/C/G/A)GT(TC-3′)(A1), 5′-A(A/C/G)(C/T)AA(A/G)GG(A/C/G/A)GT(TC-3′)(A2), 5′-A(A/C/G)(C/T)AA(A/G)GG(A/C/G/A)GT(TC-3′)(A3), and 5′-A(A/C/G)(C/T)AA(A/G)GG(A/C/G/A)GT(TC-3′)(A4). MIN6 cell poly(A)+ RNA was reverse-transcribed using oligo(DT)\textsubscript{12} and served as a PCR template. DNA amplification was achieved by a 40-cycle PCR protocol with a 30-s denaturing step at 95 °C, a 30-s annealing step at 40 or 44 °C, and a 60-s extension step at 72 °C. Reactions were performed using a mixture of sense primers S1 and S3 (2:1) or S2 and S4 (2:1), and a mixture of antisense primers A1, A2, A3, and A4 (4:2:2:1). The concentration of each mixture was 4 μM. The amplified DNA was separated on a 2% agarose gel. The purified DNA was submitted to the GenBank\textsuperscript{1} EBI Data Bank with accession number(s) D86176 and D86177.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\textsuperscript{1} EBI Data Bank with accession number(s) D86176 and D86177.

1 The abbreviations used here are: v-SNARE, vesicle membrane- and target membrane-soluble N-ethylmaleimide-sensitive factor attachment protein receptors; PI4P5K, phosphatidylinositol-4-phosphate 5-kinase; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3,4)P\textsubscript{2}, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P\textsubscript{3}, phosphatidylinositol 3,4,5-trisphosphate; HA, hemagglutinin.
fragments were ligated into pGEM-T (Promega, WI) and sequenced using an Applied Biosystems model 370 automatic sequencer. Construction and screening of the cDNA library of the pancreatic β-cell line MIN6 were performed with standard techniques as described previously (18).

Epitope Tagging and Expression of PI4PSK-Ia and -Ib by Recombinant Adenoviruses—The novel cDNAs were tagged at the amino terminus with a 9-amino acid epitope (YPYDVPDYA) derived from influenza virus hemagglutinin (HA) by in-frame insertion of the epitope sequence. Recombinant adenoviruses bearing the cDNA of one of two novel proteins with the HA-tag were constructed as described previously (19, 20). COS7 cells (1.5 × 10⁶ cells) were infected with a control virus or recombinant adenoviruses bearing these cDNAs were subjected to SDS-PAGE (10%) and were probed with these antibodies (1:1000 dilution) or anti-HA-monoclonal antibody (12CA5, 1 μg/ml). Blots were developed using ECL reagents (Amer sham, United Kingdom).

Phosphoinositide 4-Kinase Assay—Immunoprecipitation was performed using an monoclonal antibody against the HA epitope (12CA5) and protein G-Sepharose 4 First Flow (Pharmacia) and was used for the PI4PSK-Ia kinase assay. Phosphorylation of PI4PSK-Ia was carried out in an incubation medium containing a final concentration of 50 mM Tris/HCl (pH 7.5), 15 mM NaCl, 15 mM MgCl₂, 1 mM EGTA, 800 μM PtdIns(4)P, and 50 μM (γ-32P)ATP (5 μCi/tube). To investigate effects of phosphatidylinositol, PtdIns(4)P, and PtdIns(4,5)P 2 from bovine brain and phosphatidic acid were purchased from Sigma. Anti-p85 phosphatidylinositol 3-kinase antibody was used to activate these cDNAs were subjected to SDS-PAGE. The 68-kDa proteins of the two isoforms expressed in COS7 cells were immunoprecipitated using anti-HA antibody. The resulting products were identified by the anti-HA antibody or by isoform-specific antisera in cells infected with recombinant adenoviruses bearing these cDNAs (Fig. 2).

Novel cDNAs Encode Type I PI4PSK Proteins—To characterize the enzymatic activity of these isoforms, HA-tagged proteins of the two isoforms expressed in COS7 cells were immunoprecipitated using anti-HA antibody. The resulting immunocomplexes exhibited PI4PSK-Ia kinase activity (Fig. 3A). To clarify whether the observed PI4PSK-Ia kinase activity was due to PI4PSK-Ib 3-kinase or PI4PSK-Ib 5-kinase activity, a TLC was used that separates PtdIns(4,5)P 2 from PtdIns(3,4)P 2, products of the PI4PSK-Ia and PI4PSK-Ib isoform probe, respectively, in murine poly(A)⁺ RNA (Fig. 3B). Furthermore, the PI4PSK-Ia kinase activities of the Ia and the Ib isoform were increased by 11.0 ± 2.1-fold and 10.2 ± 2.2-fold, respectively, when an equimolar amount of phosphatidic acid (PA) was added to the reaction solutions (Fig. 3C). Because PA sensitivity is a major characteristic of the type I isoform (23, 24), we concluded that these novel murine cDNAs encode the type I PI4PSK-Ia 5-kinase.

Tissue Distribution of PI4PSK-I Isoforms—Northern blotting analysis was performed using cDNA probes corresponding to the sequences close to termination codons. The nucleotide sequences of these regions differed significantly between the two isoforms, allowing the detection of isoform-specific expression. A 3.5- or 4.2-kilobase mRNA was detected utilizing the Ia or the Ib isoform probe, respectively, in murine poly(A)⁺ RNA from different tissues (Fig. 4). Tissue distributions differed between the two isoforms. The Ia isoform was highly expressed in the brain and testis, but barely detectable in the liver and skeletal muscle, while the Ib isoform was found to be expressed at high levels in skeletal muscle, testis, brain, and lung tissues.

Sequence Homology—As expected, a search of protein se-

FIG. 1. Sequences of two type I PI4PSK isoforms. Two sequences shown were aligned using the computer program ALIGN. Identical residues between two proteins are represented by bold letters. Gaps are represented by dashes.
spectively, followed by detection with ECL reagents (Amersham).

The presence of phosphatidic acid (80% closed columns) was distinct from PtdIns(3,4)P2. PtdIns(3,4)P2 was generated, using PtdIns(4)P as a substrate, with p85/p110 phosphatidylinositol 3-kinase from murine brain lysates.

PtdIns(4)P kinase activity. Lysates of COS7 cells infected with adenoviruses or a control virus (Adex1CAlacZ), and the lysates (20 μg/lane) were subjected to SDS-PAGE (10%). Expressed proteins were probed with anti-HA monoclonal antibody. The immunocomplex was assayed using PtdIns(4)P as a substrate in the absence (open columns) or presence (closed columns) of phosphatidic acid (80 μM). Data are presented as means ± S.E. of three independent experiments, each performed in duplicate.

Protein of both the Iα and the Iβ isoforms migrate at 68 kDa on SDS-PAGE, suggesting that these isoforms are identical or closely related to the type Ia isoform previously described (3, 23). During the screening of the MIN6 cell cDNA library, we obtained another partial coding sequence closely homologous to the PI4P5K-Iα and Iβ isoforms. Further studies are needed to characterize the third sequence.

Whether the type I PI4P5K plays an important roles in regulated exocytosis of insulin secretory granules remains to be determined. The type I but not the type II enzyme has been demonstrated to be activated by phosphorytic acid and appears to phosphorylate PtdIns(4)P on intact membrane (31). Recent studies on regulated exocytosis have postulated that a positive feedback loop catalyzed by phospholipase D and the type I PI4P5K plays an important roles in regulated exocytosis of insulin secretory granules remains to be determined. The type I but not the type II enzyme has been demonstrated to be activated by phosphorytic acid and appears to phosphorylate PtdIns(4)P on intact membrane (31). Recent studies on regulated exocytosis have postulated that a positive feedback loop catalyzed by phospholipase D and the type I

STM7, a gene identified in the critical region for the Friedreich's ataxia locus (26), but which was recently proven not to be responsible for the disease (27). The amino acid sequence of neither the Iα nor the Iβ isoform contains regions homologous to known protein or lipid kinase domains. Both isoforms contain a sequence closely homologous to the PtdIns(4,5)P2-binding domain of chicken striated α-actinin (28): RLMKKLEH-SWK and RFVKKLEHSWK, in the Iα and the Iβ isoforms, respectively.

DISCUSSION

We have identified two type I isoforms of PI4P5K, designated PI4P5K-Iα and PI4P5K-Iβ, in this study. PI4P5K catalyzes the formation of PtdIns(4,5)P2 from PtdIns(4)P. PtdIns(4,5)P2 has been demonstrated to be an important molecule in various cellular processes, including inositol 1,4,5-trisphosphate mediated Ca2+ mobilization (4), actin filament reorganization (5, 6), and exocytosis (7). Co-existence of at least three isoforms of the PI4P5K (one previously cloned isoform (type II or C) and two type I) in an insulin-secreting cell line, MIN6, suggests that these isoforms have distinct roles in various cellular processes. These isoforms may reside in different subcellular compartments and that spatially segregated synthesis or metabolism of PtdIns(4,5)P2 may be linked to different cellular functions. While these novel isoforms show approximately 75% identity in their central regions, the amino-terminal or carboxyl-terminal sequences are different. It is possible that the amino- and carboxyl-terminal regions have isoform-specific functions. Further studies of these isoforms are needed; of particular interest is their subcellular localization and possible association with other molecules, such as Rac1 (29) and Rho (30).

Proteins of both the Iα and the Iβ isoform migrate at 68 kDa on SDS-PAGE, suggesting that these isoforms are identical or closely related to the type Ia isoform previously described (3, 23). During the screening of the MIN6 cell cDNA library, we obtained another partial coding sequence closely homologous to the PI4P5K-Iα and Iβ isoforms. Further studies are needed to characterize the third sequence.

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PI4P5K is involved in exocytotic processes (8), which may form a microdomain enriched in negatively charged phospholipids on the vesicle membrane. Our demonstration of several isoforms of the type I PI4P5K in insulin-secreting cells (MIN6), together with a previous demonstration of phospholipase D in pancreatic islets (32), raises the possibility that such a positive feedback loop may operate in insulin-secreting cells. Furthermore, it was previously reported that glucose could cause activation of phosphatidylinositol metabolism, which was partly ascribed to increased de novo synthesis of PA and polyphosphoinositide in pancreatic islets (10, 11). These observations suggest that activation of phosphatidylinositol metabolism, in concert with membrane depolarization, may serve as a link between glucose metabolism and exocytosis of insulin secretory granules. Molecular identification of the type I PI4P5K isoforms will provide essential information for the precise role of phosphatidylinositol metabolism in exocytosis of insulin-containing dense core vesicles.

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