Human Pancreatic Islets Express mRNA Species Encoding Two Distinct Catalytically Active Isoforms of Group VI Phospholipase A$_2$ (iPLA$_2$) That Arise from an Exon-skipping Mechanism of Alternative Splicing of the Transcript from the iPLA$_2$ Gene on Chromosome 22q13.1*

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An 85-kDa Group VI phospholipase A$_2$ enzyme (iPLA$_2$) that does not require Ca$^{2+}$ for catalysis has recently been cloned from three rodent species. A homologous 88-kDa enzyme has been cloned from human B-lymphocyte lines that contains a 54-amino acid insert not present in the rodent enzymes, but human cells have not previously been observed to express catalytically active iPLA$_2$ isoforms other than the 88-kDa protein. We have cloned cDNA sequences that encode two distinct iPLA$_2$ isoforms from human pancreatic islet RNA and a human insulinoma cDNA library. One isoform is an 85-kDa protein (short isoform of human iPLA$_2$ (SH-iPLA$_2$)) and the other an 88-kDa protein (long isoform of human iPLA$_2$ (LH-iPLA$_2$)). Transcripts encoding both isoforms are also observed in human promonocytic U937 cells. Recombinant SH-iPLA$_2$ and LH-iPLA$_2$ are both catalytically active in the absence of Ca$^{2+}$ and inhibited by a bromoenol lactone suicide substrate, but LH-iPLA$_2$ is activated by ATP, whereas SH-iPLA$_2$ is not. The human iPLA$_2$ gene has been found to reside on chromosome 22 in region q13.1 and to contain 16 exons represented in the LH-iPLA$_2$ transcript. Exon 8 is not represented in the SH-iPLA$_2$ transcript, indicating that it arises by an exon-skipping mechanism of alternative splicing. The amino acid sequence encoded by exon 8 of the human iPLA$_2$ gene is proline-rich and shares a consensus motif of $P_X^9P_X^9HIPX_3^7N_X^4Q$ with the proline-rich middle linker domains of the Smad proteins DAF-3 and Smad4. Expression of mRNA species encoding two active iPLA$_2$ isoforms with distinguishable catalytic properties in two different types of human cells demonstrated here may have regulatory or functional implications about the roles of products of the iPLA$_2$ gene in cell biologic processes.

Phospholipases A$_2$ (PLA$_2$) catalyze hydrolysis of sn-2 fatty acid substituents from glycerophospholipid substrates to yield a free fatty acid and a 2-lysophospholipid (1–7). PLA$_2$ is a diverse group of enzymes, and the first well characterized members have low molecular masses (approximately 14 kDa), require millimolar [Ca$^{2+}$] for catalytic activity, and function as extracellular secreted enzymes (sPLA$_2$) (3, 6). The first cloned PLA$_2$ that is active at [Ca$^{2+}$] achieved in the cytosol of living cells is an 85-kDa protein classified as a Group IV PLA$_2$ and designated cPLA$_2$ (3, 5). This enzyme is induced to associate with its substrates in membranes by rises in cytosolic [Ca$^{2+}$] within the range achieved in cells stimulated by extracellular signals that induce Ca$^{2+}$ release from intracellular sites or Ca$^{2+}$ entry from the extracellular space, is also regulated by phosphorylation, and prefers substrates with sn-2 arachidonoyl residues (5).

Recently, a second PLA$_2$ that is active at [Ca$^{2+}$] that can be achieved in cytosol has been cloned (8–10). This enzyme does not require Ca$^{2+}$ for catalysis, is classified as a Group VI PLA$_2$, and is designated iPLA$_2$ (3, 4). The iPLA$_2$ enzymes cloned from hamster (8), mouse (9), and rat (10) cells represent species homologs and all are 85-kDa proteins containing 752 amino acid residues with highly homologous (approximately 95% identity) sequences. Each contains a GXXG lipase consensus motif and eight stretches of a repeating motif homologous to a repetitive motif in the integral membrane protein-binding domain of ankyrin (8–10). The substrate preference of these iPLA$_2$ enzymes varies with the mode of presentation (8), but each is inhibited (8–10) by a bromoenol lactone (BEL) suicide substrate (11, 12) that is not an effective inhibitor of sPLA$_2$ or cPLA$_2$ enzymes at comparable concentrations (4, 11–14).

Proposed functions for iPLA$_2$ include a housekeeping role in phospholipid remodeling that involves generation of lysophospholipid acceptors for arachidonic acid incorporation into P388D1 macrophage-like cell phospholipids (4, 15, 16). Signal transduction roles for iPLA$_2$ in generating substrate for leukotriene biosynthesis (17) and lipid messengers that regulate ion channel activity (10, 18, 19) and apoptosis (20) have also been suggested. Recent observations with human iPLA$_2$ suggest that the enzyme might serve distinct functions in different cells that involve regulatory interactions among splice variants (17, 21). Human iPLA$_2$ cloned from B-lymphocyte lines and testis differs from iPLA$_2$ cloned from cells of rodent species in that it

situ hybridization; IPTG, isopropyl-1-thio-b-D-galactopyranoside; kb, kilobase pair(s); PCR, polymerase chain reaction; RT, reverse transcription; Sf9, Spodoptera frugiperda, type 9; PLA$_2$, phospholipase A$_2$; cPLA$_2$, Group IV PLA$_2$; iPLA$_2$, Group VI PLA$_2$; sPLA$_2$, secretory PLA$_2$; LH-iPLA$_2$, long isoform of human iPLA$_2$; SH-iPLA$_2$, short isoform of human iPLA$_2$.
is an 88-kDa rather than an 85-kDa protein and contains a 54-amino acid insert interrupting the eighth ankyrin repeat (21). The human B-lymphocyte iPLA2 sequence is otherwise highly homologous to hamster, mouse, and rat sequences and includes the seven other ankyrin-like repeats and a GXXG lipase sequence (21). Catalytically active iPLA2 other than the 88-kDa isoform have not yet been observed in human cells (21).

Human B-lymphocyte lines do express truncated, inactive iPLA2 sequences that contain the ankyrin repeat domain but lack the catalytic domain and are thought to arise from alternative splicing of the transcript (21). Co-expression of the truncated sequences with full-length human iPLA2 attenuates catalytic activity (21). Because the active form of iPLA2 is an oligomeric complex (8, 22) that may result from subunit associations through ankyrin repeat domains (8), this suggests that formation of hetero-oligomeric complexes represents a means to regulate iPLA2 activity (21). That mechanisms of iPLA2 regulation differ among human cell types is suggested by the fact that stimuli that induce iPLA2-catalyzed arachidonate release and leukotriene production in human granulocytes fail to induce these events in human lymphocyte lines, even though both classes of cells express iPLA2 and leukotriene biosynthetic enzymes (17, 21).

One human cell type in which iPLA2 may be biomedically important is the pancreatic islet beta cell. Impaired beta cell survival and signaling functions underlie development of types I and II diabetes mellitus, respectively; these are the most prevalent human endocrine diseases. In rodent islets, iPLA2 has been proposed to play a signaling role in glucose-induced insulin secretion (8, 23–25) and in experimentally induced beta cell apoptosis (26). Human islets express a BEL-sensitive PLA2 activity that does not require Ca2+ (27, 28), but iPLA2 mRNA has not been demonstrated in human islets. We have cloned human beta cell iPLA2 cDNA here and find that human islets express mRNA species encoding two iPLA2 isoforms with different sizes (85 and 88 kDa) and catalytic properties. We have also determined the human iPLA2 gene structure and its chromosomal location and find that the transcript encoding the short isoform arises from an exon-skipping mechanism of alternative splicing.

**EXPERIMENTAL PROCEDURES**

**Materials**—The compounds [3H]dCTP (3000 Ci/mmol), [35S]dATPS (1000 Ci/mmol), and 1,2- or 1-palmitoyl-2-[14C]arachidonoyl-phosphatidylethanolamine (50 mCi/mmol) and ECL detection reagents were obtained from Amersham Pharmacia Biotech, and the BEL (E)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one) iPLA2 suicide substrate was obtained from BIORAD (Plymouth Meeting, PA). A human placental genomic DNA library in lambda FIX II was obtained from Stratagene (La Jolla, CA). Human promonocytic U937 cells (30) were obtained from American Type Culture Collection (Manassas, VA) and cultured as described (20, 31). Sources of other common materials are identified elsewhere (10, 14, 23–25, 28).

Cloning cDNA Species Containing iPLA2 Sequence from a Human Insulinoma Cell cDNA Library—Rat islet iPLA2 cDNA was isolated (10), labeled with [35S]dATP, and used to screen a human insulinoma cell cDNA library (32) provided by Dr. Alan Permatt of Washington University. Insert sizes in clones that hybridized with the probe were determined by digestion with restriction endonucleases, and their sequences were determined from the double strand (33). Two cDNA species were obtained that contained about 1.80 and 1.59 kb, respectively, of the insulinoma cell cDNA that had been treated with NcoI. The fragments were subcloned into pBluescript SK. Fragments from RT-PCR of human islet RNA contained about 1.59 and 1.79 kb of DNA. The RT-PCR was performed with human islet RNA and specific primers.

**Isolation of RNA from Human Islets and Human Promonocytic U937 Cells, Reverse Transcription, and Polymerase Chain Reaction—**Islets were isolated from human pancreata in the Washington University Diabetes Research and Training Center (34) and cultured as described (35). Total RNA was isolated from human islets and promonocytic U937 cells and first-strand cDNA prepared by reverse transcription (RT) using standard procedures (36). PCR products were performed under described conditions (10), and products were analyzed by agarose gel electrophoresis (36). Primers used to generate the 5′ portion of human iPLA2 cDNA were sense (5′-GATGACGTCTTTGGGACCTGGG-3′), anti-sense (5′-TACGCATACCTGGTTGTTCC-3′), and nested antisense (5′-AATGCGGACCGGCAATGATC-3′). Two distinct cDNA fragments were subcloned into pBluescript SK. Fragments from RT-PCR of human islet RNA contained the iPLA2 5′-coding sequence and were released from plasmids with EcoRI and NcoI. Products were isolated by agarose gel electrophoresis and ligated with a plasmid containing the 3′-end of human iPLA2 cDNA that had been treated with NcoI. Ligation product plasmids were used to transform bacterial host cells and sequenced. The resultant cDNA species contained complete coding sequences of human iPLA2 isoforms and were inserted into appropriate vectors for expression and used to prepare [35S]labeled human iPLA2 cDNA for genomic screening.

**Preparation of cDNA Species Containing the Complete Human Islet iPLA2 Coding Sequence—**The cDNA species obtained from screening the human insulinoma cell cDNA library overlapped at their 5′-ends with 3′-ends of cDNA fragments from RT-PCR of human islet RNA, and the coding region contained an NcoI site. The fragments were subcloned into pBluescript SK. Fragments from RT-PCR of human islet RNA contained the iPLA2 5′-coding sequence and were released from plasmids with EcoRI and NcoI. Products were isolated by agarose gel electrophoresis and ligated with a plasmid containing the 3′-end of human iPLA2 cDNA that had been treated with NcoI. Ligation product plasmids were used to transform bacterial host cells and sequenced. The resultant cDNA species contained complete coding sequences of human iPLA2 isoforms and were inserted into appropriate vectors for expression and used to prepare [32P]labeled human iPLA2 cDNA for genomic screening.

**Bacterial Expression of Recombinant Human Islet iPLA2 Isoforms—**The cDNA species encoding full-length human islet iPLA2 isoforms were cloned in-frame into the EcoRI and XhoI sites of pBac28c (Novagen). The constructs were analyzed by restriction endonuclease digestion, sequenced, and transformed into bacterial expression host BL21(DE3) (Novagen). Cells transformed with pET28c without insert were negative controls. Protein expression was induced by treating cells with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and assayed by SDS-polyacrylamide gel electrophoresis analyses with Coomassie Blue staining and by immunoblotting under described conditions (10) with a rabbit polyclonal antibody against recombinant rat islet iPLA2.

**Eukaryotic Expression of Recombinant Human Islet iPLA2 Isoforms—**The Spodoptera frugiperda (Sf9) insect cell-baculovirus system was used to express other PLAs enzymes in catalytically active forms (37, 38) as previously described by Braun (39) for expressing human iPLA2. We initially constructed recombinant baculovirus with human islet iPLA2 isoform cDNA inserts. Infection and culture were performed under defined conditions (47). At 48 h after infection, Sf9 cells were collected by centrifugation, washed, resuspended in buffer (250 mM sucrose, 25 mM imidazole, pH 8.0), and disrupted by sonication. Cytosolic and membranous fractions were prepared by sequential centrifugations (10,000 × g for 10 min and 100,000 × g for 60 min) and used for PLAs activity assays.

**Phospholipase A2 Activity Assays—**The protein content of Sf9 cell cytosolic and membranous fractions was determined by Bio-Rad assay, and iPLA2 activity was measured in aliquots (approximately 20 μg of protein) added to assay buffer (200 mM Tris-HCl, pH 7.0; total assay volume, 200 μl) containing 5 mM EGTA with or without 1 mM ATP. Some aliquots were pretreated (2 min) with BEL (10 μM) before the assay. Reactions were initiated by injecting substrate (1,2- or 1-palmitoyl-2-[14C]arachidonoyl-phosphatidylethanolamine; specific activity, 50 Ci/ mol; final concentration, 5 μM) in ethanol (5 μl). Assay mixtures were incubated (3 min at 37 °C), and reactions were terminated by adding butanol (0.1 ml) and vortexing. After centrifugation (2000 × g for 4 min), products in the butanol layer were analyzed by silica gel TLC in hexane/ethyl ether/acetonic acid (80:20:1). The TLC region containing free arachidonic acid (Rf, 0.58) was scraped into vials, and its 14C content was determined.

**Cloning Human iPLA2 Genomic DNA Fragments, Determination of Intron-Exon Boundaries, and Estimation of Intron Size—**A [32P]-labeled human islet iPLA2 cDNA was used to screen a human placental Lambda FIX II genomic DNA library (Stratagene). Clones that hybridized with the probe were isolated and plaque-purified, and the lambda DNA fragments containing genomic DNA inserts were purified by standard procedures (36). Inserts were excised with NcoI and subcloned into a pBluescript SK plasmid for restriction site mapping. Sequences of intron-exon boundaries were determined by comparing sequences of genomic DNA and cDNA. Intron sizes were estimated from lengths of PCR products from reactions using genomic DNA as template and primers that hybridize to sequences in adjacent exons.

**Chromosomal Mapping of Human iPLA2 Gene by Fluorescence in
**RESULTS**

**Characterization of iPLA<sub>2</sub> cDNA from Human Islets—**To determine whether human pancreatic islet beta cells express mRNA species encoding iPLA<sub>2</sub>, a human insulinoma cell cDNA library (32) was screened with a 32<sup>P</sup>-labeled rat iPLA<sub>2</sub> cDNA (10) probe. Two clones (INS-C1 and INS-C2) of about 1.59 and 1.80 kb in length, respectively, hybridized to the probe and were sequenced. Both clones contained identical 3'-sequences that included a presumptive polyadenylation sequence and a poly(A) tail, and their sequences were identical except for additional 5'-sequence in the longer clone not contained in the shorter clone. Alignment with the rat iPLA<sub>2</sub> cDNA sequence indicated that the clones contained the 3'-end of human iPLA<sub>2</sub> cDNA, but neither contained the full 5'-coding sequence (Fig. 1). RNA from human islets was therefore used as template in RT-PCRs with primers designed from the 5'-sequence of rat iPLA<sub>2</sub> cDNA and from sequence in INS-C1 and INS-C2 cDNAs. The primers were designed to amplify cDNA from the initiator methionine codon at the 5'-end through the region of sequence at the 3'-end recognized by primers designed from INS-C1 and INS-C2 sequences. A nested primer approach was employed in 3'-end primers to verify specificity of amplification products. When used with the same 5'-primer, one of the 3'-primers was expected to yield a longer product than the other.

RT-PCRs with a given set of these primers using human islet mRNA species encoding iPLA<sub>2</sub>, a human insulinoma cell cDNA isoforms of human iPLA<sub>2</sub> are designated LH-iPLA<sub>2</sub> and SH-iPLA<sub>2</sub>, respectively. These cDNA species were prepared by NcoI digestion and ligation of the insulinoma cell cDNA fragment and one of the two RT-PCR products derived from human islet RNA. R-iPLA<sub>2</sub>, rat iPLA<sub>2</sub> cDNA. The region of the 162-bp insert that distinguishes LH-iPLA<sub>2</sub> from SH-iPLA<sub>2</sub> is indicated by the **black bar**. The remainder of the coding sequence is indicated by **shaded bars**. The lighter shading represents human iPLA<sub>2</sub> coding sequence; the darker shading represents rat iPLA<sub>2</sub> coding sequence.

![Diagram showing the location of the recognition site for the restriction endonuclease NcoI that is contained in the region of overlap between the insulinoma cell cDNA fragments and the human islet RNA RT-PCR products.](image)

**Fig. 1.** Summary of cDNA fragments used to construct cDNA species containing the complete coding sequences of human islet iPLA<sub>2</sub> isoforms. The two cDNA clones obtained by screening the human insulinoma cell cDNA library that contain the 3'-sequence of the human islet iPLA<sub>2</sub> cDNA are designated INS-C1 and INS-C2. The RT-PCR products obtained using human islet RNA as template that contain the 5'-end of the human islet iPLA<sub>2</sub> coding sequence (see Fig. 2) are designated human islet PCR long fragment and human islet PCR short fragment. Arrows indicate the location of the recognition site for the restriction endonuclease NcoI that is contained in the region of overlap between the insulinoma cell cDNA fragments and the human islet RNA RT-PCR products. The cDNA species containing the complete coding sequence of the long and short isoforms of human iPLA<sub>2</sub> are designated LH-iPLA<sub>2</sub> and SH-iPLA<sub>2</sub>, respectively. These cDNA species were prepared by NcoI digestion and ligation of the insulinoma cell cDNA fragment and one of the two RT-PCR products derived from human islet RNA. R-iPLA<sub>2</sub>, rat iPLA<sub>2</sub> cDNA. The region of the 162-bp insert that distinguishes LH-iPLA<sub>2</sub> from SH-iPLA<sub>2</sub> is indicated by the **black bar**. The remainder of the coding sequence is indicated by **shaded bars**. The lighter shading represents human iPLA<sub>2</sub> coding sequence; the darker shading represents rat iPLA<sub>2</sub> coding sequence.

**Fig. 2.** Agarose gel electrophoretic analyses of products of RT-PCRs performed with human islet RNA or U937 cell RNA as template and primers designed to amplify the 5'-end of human iPLA<sub>2</sub> cDNA. RT-PCRs were performed with RNA isolated from human islets from two different donors (A) or from two different preparations of human promonocytic U937 cell RNA (B). In experiments shown in lanes 1, 2, and 5 were performed with RNA from islets from donor 1, and experiments shown in lanes 3, 4, and 6 were performed with RNA from islets from donor 2. Reverse transcriptase was omitted from the reactions analyzed in lanes 1 and 3 to exclude contamination from genomic DNA in the human islet RNA preparations. In reactions analyzed in lanes 1–4 (A), a set of PCR primers was used that was expected to yield a single 1.65-kb product, based on the rat islet iPLA<sub>2</sub> cDNA sequence. In reactions analyzed in lanes 5 and 6 (A), the same 5'-primer was used as in the reactions analyzed in lanes 1–4, but a different 3'-primer was used that was expected to yield a shorter product, based on the rat iPLA<sub>2</sub> cDNA sequence. The sequences of the 5'-primer and of the two 3'-primers used in these reactions are specified under “Experimental Procedures.” In B, experiments shown in lanes 1 and 2 were performed with RNA from U937 cell preparation 1, and experiments shown in lanes 3 and 4 were performed with RNA from U937 cell preparation 2. Reverse transcriptase was omitted from the reactions analyzed in lanes 1 and 3 (B). In reactions analyzed in lanes 1–4 (B), the set of PCR primers was the same as that in lanes 1–4 of A. Both of the RT-PCR products visualized in lanes 2 and 4 (B) were subcloned and sequenced, and the results were identical to those for the products in lanes 2 and 4 of A.

RNA as template yielded two products (Fig. 2A). The experiments shown in lanes 1–4 of the agarose gel electrophoretic analysis of the RT-PCR products (Fig. 2A) were performed with a primer set expected to yield a product of 1.65 kb in length based on the rat iPLA<sub>2</sub> cDNA sequence. Lanes 1 and 3 represent PCRs performed without an RT step to exclude contamination with genomic DNA, and no amplification products were observed. Lanes 2 and 4 represent RT-PCRs performed with two different preparations of human islet RNA, which both yielded two products. The more intensely staining product exhibited the 1.65-kb length expected from the rat iPLA<sub>2</sub> cDNA sequence. There was also a less intensely staining band at 1.85 kb. Lanes 5 and 6 represent RT-PCRs performed with human RNA as template, the same 5'-primer used in lanes 1–4, and a nested 3'-end primer expected to yield a shorter product...
than that obtained with the 3'-end primer used in the experiments shown in lanes 1–4. Two products were again observed. The more intensely staining band exhibited the length expected based on the rat iPLA2 cDNA sequence, and it was accompanied by another band about 0.2 kb longer.

The 1.65- and 1.85-kb human islet RT-PCR products in Fig. 2A, lanes 2 and 4, were subcloned and sequenced. Each contained a 5'-coding sequence that specified an amino acid sequence highly homologous to the N-terminal portion of rat iPLA2. The nucleotide sequences of the two human iPLA2 mRNA species were identical except for a 162-bp insert in the longer product that was not contained in the shorter product. This insert did not interrupt the reading frame and encoded a 54-amino acid insert in the eighth ankyrin repeat of the iPLA2 amino acid sequence. Similar RT-PCRs using RNA from human U937 promonocytic cells as template and the primer set employed in Fig. 2A, lanes 1–4, indicated that U937 cells also express two distinct iPLA2 mRNA species (Fig. 2B), and the lengths of the two RT-PCR products corresponded exactly to those from human islet RNA. The relative intensities of the two products differed, however, and staining of the band for the longer product was more intense than that for the shorter product when U937 cell RNA was used as template. The converse was true with human islet RNA. The U937 cell RT-PCR products were subcloned and sequenced and were identical to the products from human islet RNA. RT-PCRs in Fig. 2 are analogous to competitive PCR (10, 66, 67) and involve amplification of two distinct cDNA species from the same primer set in the same reaction mixture. As with competitive RT-PCR, relative abundances of reaction products in Fig. 2 may reflect the relative abundances of different cDNA species in the original reaction mixture.

These findings indicate that some human cells express mRNA species that encode two distinct isoforms of iPLA2. While these experiments were in progress, cloning of human iPLA2 from lymphocyte lines and testis was reported (21). That report identified only one human mRNA species that encoded a full-length iPLA2 sequence, and it corresponded to the longer isoform predicted from our results. No mRNA species corresponding to the shorter human iPLA2 isoform predicted from our results was observed in human B-lymphocyte lines or human testis, but two mRNA species, thought to represent alternative splicing products, were observed that encoded truncated iPLA2 variants that contained the ankyrin repeat region but lacked the catalytic domain (21). We sought evidence for expression mRNA encoding these truncated iPLA2 variants in human islets and human U937 promonocytic cells and observed them in U937 cells but not in islets (not shown), suggesting that there is heterogeneity among human cells in expression of products of the iPLA2 gene.
Fig. 3 illustrates nucleotide and deduced amino acid sequences predicted from our results for cDNAs encoding the long and short isoforms of human iPLA₂. The predicted amino acid sequence for the long isoform differs from that for the short isoform only by the presence of a 54-amino acid insert interrupting the eighth ankyrin repeat. The short isoform is highly homologous to the hamster, mouse, and rat iPLA₂ sequences, all of which also lack the 54-amino acid insert (8–10). This insert is proline-rich, and a BLAST search revealed similarities to the proline-rich middle linker domain of the DAF-3 Smad protein from \textit{Caenorhabditis elegans} (42), which is most closely related (42) to mammalian Smad4 (43). The proline-rich middle linker region of Smad4 shares a $P\underbrace{X\cdots X}_8HHP\underbrace{X\cdots X}_4Q$ motif with the corresponding region of DAF-3 and the proline-rich insert in the long human iPLA₂ isoform.

In Fig. 4, residues that are identical among the three sequences are indicated by dark boxes, and residues with chemically similar side chains are indicated by light boxes. The Smad4 middle linker domain mediates protein interactions with signaling partners (43), is located near the center of the protein, and separates an N-terminal MH1 domain with DNA binding activity from a C-terminal MH2 domain with transcriptional activity (54). The proline-rich insert in the long iPLA₂ isoform is also located near the center of the protein and separates an N-terminal domain with protein binding activity from a C-terminal catalytic domain.

Bacterial Expression of Recombinant Human Islet iPLA₂ Isoforms—To demonstrate that the human islet iPLA₂ isoform cDNA species encoded proteins of expected sizes, they were subcloned into expression vector pET-28c, and the resultant constructs were used to transform bacterial host BL21(DE3). Expression of proteins encoded by cDNA inserts was induced by IPTG treatment, and proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 5). In IPTG-treated cells, proteins of the expected sizes, 85 (lane 2) or 88 (lane 4) kDa, were produced from cDNA for SH-iPLA₂ or LH-iPLA2, respectively, in much greater abundance than in non-IPTG-treated cells (lanes 1 and 3). Both proteins were recognized by a polyclonal antibody against rat iPLA₂ (not shown).

Eukaryotic Expression of Recombinant Human Islet iPLA₂ Isoforms—To determine whether human islet iPLA₂ isoform cDNA species encoded catalytically active enzymes, a baculovirus vector-Sf9 cell system was used in which other PLA₂ enzymes have been expressed (38, 39). Recombinant baculovirus that contained inserts encoding LH-iPLA₂ or SH-iPLA₂ were used for infection, and subcellular fractions from infected cells were assayed for iPLA₂ activity. Uninfected Sf9 cells exhibited no detectable iPLA₂ activity, but such activity was observed in cytosolic and membranous fractions of cells infected with baculovirus that contained cDNA inserts encoding either human iPLA₂ isoform (Fig. 6). The iPLA₂ activities expressed in cells infected with baculovirus that contained cDNA encoding human iPLA₂ isoform were inhibited by the iPLA₂ suicide substrate (4, 8–12) BEL (Fig. 6). We believe this to be the first demonstration that recombinant human iPLA₂ is inhibited by BEL, as this issue was not examined in a recent report on human iPLA₂ cloned from lymphocyte lines and testis (21). Activities of recombinant LH-iPLA₂ and SH-iPLA₂ were affected differently by 1 mM ATP (Fig. 6). ATP exerted a stimulatory effect on LH-iPLA₂ activities in cytosol or membranes.
similar side chains are observed in two or three of the sequences are designated with Greek letters (Fig. 6).

bacterial expression vector pET-28c (Novagen). The pET28-iPLA2 construct was induced by treating the cells with IPTG, and proteins expressed by induced (lanes 2 and 4) and noninduced (lanes 1 and 3) cells were compared by SDS-polyacrylamide gel electrophoresis analyses with Coomassie Blue staining. The expected molecular mass of the long isoform of human iPLA2 is 88 kDa (lane 2), and that of the long isoform of human iPLA2 is 88 kDa (lane 4).

but did not affect SH-iPLA2 activities. ATP has been reported to stimulate iPLA2 activities from rat islets (10) and murine P388D1 cells (9) but not to affect the iPLA2 activity of Chinese hamster ovary cells (6). These findings indicate that cDNA species for both LH-iPLA2 and SH-iPLA2 encode catalytically active enzymes and that catalytic properties of the two human iPLA2 isoforms differ. The experiments also suggest that the ratio of membranous to cytosolic activity may differ somewhat for LH-iPLA2 and SH-iPLA2 under these assay conditions (Fig. 6).

Characterization of the Human iPLA2 Gene—To explore the basis for producing human islet mRNA species that encode the two distinct iPLA2 isoforms, the structure of the human iPLA2 gene was determined. A 32P-labeled LH-iPLA2 cDNA was used as probe to screen a human Lambda FIX II genomic DNA library. Eight genomic clones with overlapping regions of sequence were isolated and analyzed by Southern hybridization and restriction endonuclease digestion. Fig. 7 is a schematic representation of the human iPLA2 gene structure. The cloned sequence spans over 60 kb of DNA and includes 16 exons representing 5' -untranslated region, the entire coding sequence, and 3'-untranslated region of the LH-iPLA2 transcript. Introns sizes were estimated from the length of PCR fragments produced by using genomic DNA as template and primers that hybridize with sequences in adjacent exons. The sequences of intron-exon boundaries were determined by comparing the sequences of genomic DNA and cDNA. Table I summarizes the sequences at the 3' acceptor sites and the 5' donor sites at these boundaries. In each case, the intron sequence at the 5'-boundary of the exon ended in the dinucleotide AG and that at the 3'-boundary of the exon began with the dinucleotide GT, conforming to recognized rules for sequences at such junctions (29).
FIG. 7. Schematic representation of the structure of the human iPLA₂ gene and its restriction endonuclease map. The line at the top of the diagram indicates the scale in kb. There is an interruption in the scale between 0 and 25 kb because of the long length of the first intron. The locations of cleavage sites for restriction endonucleases are illustrated beneath the scale line. Below the summary of endonuclease sites, the location of the coding regions for the gene is indicated. The regions of the gene that are included in four recognized splice-variant products iPLA₂ gene are illustrated schematically in the bottom four lines. The human islet LH-iPLA₂ isoform transcript contains exons 1–7 and 9–16 but not exon 8 or alternate exons 8b or 9b. Two iPLA₂ splice variants have been reported by others in human B-lymphocyte lines (21). The transcripts for these variants contain intron sequences that result in premature stop codons and encode truncated forms of iPLA₂ that contain the ankyrin repeat domain but lack the catalytic domain. These variants are designated human B-lymphocyte ankyrin-iPLA₂-1 and human B-lymphocyte ankyrin-iPLA₂-2. The open rectangles reflect the sites of the intron sequences that are contained in these truncation variants, and the location of these intron sequences in the iPLA₂ gene are designated by sites 8b and 9b.

Alternatively Spliced mRNA Species Encoding Long and Short Isoforms of Human iPLA₂—The 54-amino acid insert interrupting the last ankyrin repeat in the LH-iPLA₂ isoform corresponds exactly to the amino acid sequence encoded by exon 8 of the human iPLA₂ gene. This indicates that mRNA encoding the SH-iPLA₂ isoform arises from an exon-skipping mechanism of alternative splicing (29) in transcription of the iPLA₂ gene. Different mechanisms of alternative splicing are involved in producing iPLA₂ truncation variants observed in human B-lymphocyte lines (21), as illustrated in Fig. 7. The variants contain additional sequence arising from introns that results in premature stop codons, and they encode truncated proteins that contain the ankyrin repeat domain but lack the iPLA₂ catalytic domain. The locations within the human iPLA₂ gene of intron sequences in the transcripts for the truncation variants were determined from PCR experiments using primers designed from the identified exon sequences and from the published (21) sequences of cDNA species encoding the truncation variants. The truncation variant human B-lymphocyte ankyrin-iPLA₂-1 contains sequence from the intron between exons 9 and 10. The truncation variant human B-lymphocyte ankyrin-iPLA₂-2 contains sequence from two intron regions. The first resides between exons 8 and 9 and the second between exons 9 and 10. The second region of intron sequence occurs in transcripts encoding each of the truncation variants (Fig. 7). Table I indicates the sequences at the intron-exon junctions for these alternate exons.

Chromosomal Localization of Human iPLA₂ Gene—To determine the location of the iPLA₂ gene on human chromosomes, a clone identified in screening the human genomic DNA library with LH-iPLA₂ cDNA was biotinylated to generate a probe for FISH experiments with human lymphocyte chromosomes (40, 41). Using this probe, 91 of 100 examined mitotic figures exhibited fluorescent signals on one pair of chromosomes (Fig. 8), indicating a hybridization efficiency of 91%. The human chromosomes were identified by their DAPI banding patterns (40, 41), and these patterns were correlated with the site of fluorescent signals from biotinylated probe. Such comparisons indicated that the iPLA₂ gene resides on human chromosome 22. A detailed positional assignment achieved from analyses of 10 photographs indicated that the iPLA₂ gene resides in region q13.1 of chromosome 22 (Fig. 8). No other loci of hybridization of the probe were observed.

DISCUSSION

Our results indicate that human pancreatic islets express mRNA species encoding two distinct, catalytically active isoforms of iPLA₂ that are distinguishable by size and by their susceptibility to activation by ATP. These two mRNA species are also observed in human U937 promonocytic cells. These two human iPLA₂ isoforms differ only by the presence of a 54-amino acid insert in the longer isoform that is absent from the
demonstrate expression of iPLA2 mRNA by human islets or identified in human cells, and we believe our studies are the first to identified in screening the human genomic DNA library with the iPLA2 cDNA was biotinylated to generate a probe for FISH experiments with exon-skipping mechanism of alternative splicing. The mRNA of recombinant human iPLA2 enzymes.

... induced by a BEL-sensitive phospholipase (47), such as iPLA2 (20). Our demonstration that U937 cells express iPLA2 mRNA indicates that this PLA2 activity may reside in the iPLA2 protein. Anti-Fas antibodies induce U937 cell apoptosis and hydrolysis of arachidonic acid from cell membranes (20). During this process, cPLA2 is proteolytically inactivated by caspases, but iPLA2 activity is preserved (20). Inhibitors of iPLA2 both suppress Fas antibody-induced arachidonate release from U937 cells and retard cell death (20), suggesting that iPLA2 may participate in apoptosis.

Similarly, stimuli that induce Ca2+ store depletion in islet beta cells induce apoptosis by a mechanism that does not require a rise in cytosolic [Ca2+] but that does require hydrolysis of arachidonic acid from membrane phospholipids and its conversion to 12-lipoxygenase metabolites (28). Ca2+ store depletion-induced hydrolysis of arachidonic acid from islet phospholipids also does not require a rise in cytosolic [Ca2+] and is mediated by a BEL-sensitive phospholipase (47), such as iPLA2. Although phosphatidate phosphohydrolase is also inhibited by BEL (48), the phosphatidate phosphohydrolase in-
hibitor propranolol (49) does not block Ca\(^{2+}\) store depletion-induced release of arachidonate from islet phospholipids (47), suggesting that iPLA\(_2\) may mediate this phenomenon. Interleukin-1 also induces accumulation of non-esterified arachidonic acid and its 12-lipoxygenase products in islets by a BEL-sensitive mechanism (50), and interleukin-1 induces apoptosis of human islet beta cells through Fas-mediated events (51). In the context of these observations, our findings that human islets express iPLA\(_2\) mRNA raise the possibility that iPLA\(_2\) might participate in Fas-mediated apoptosis in human beta cells in a manner similar to that in U937 cells (20). Beta cell apoptosis is thought to contribute to development of diabetes mellitus (52).

The amino acid sequence of the insert that distinguishes the long and short isoforms of human iPLA\(_2\) is of interest in the context of the potential involvement of iPLA\(_2\) in apoptosis. This insert shares a PX_\(X_8\)HHPX_\(12\)NX_Q consensus motif with the proline-rich middle linker domains of the \(C\). \(e\)legans Smad protein DAF-3 (42) and the mammalian protein Smad4 (43). Smad proteins participate in controlling cell proliferation and apoptosis and form hetero-oligomers with signaling partners (54), via the proline-rich middle linker domain in the case of Smad4 (43). Smad4 and Smad2 are products of tumor-suppressor genes that are deleted or mutated in some human carcinomas (54–58). Studies of allelic losses in human breast and head and neck carcinomas indicate that a tumor suppressor gene(s) resides on human chromosome 22q13.1 (59, 60), which is the chromosomal location of the human iPLA\(_2\) gene. If iPLA\(_2\) does participate in apoptosis (20, 26), it might exert tumor suppressor activity.

The active form of iPLA\(_2\) appears to be an oligomer of interacting protein subunits. Radiation inactivation studies of iPLA\(_2\) activity in crude cytosol indicate a size of 337 kDa for the active complex (22). The iPLA\(_2\) activity in crude cytosol also migrates with an apparent molecular mass of 250–350 kDa on gel filtration chromatography (8, 22, 61), and this is also the case for the iPLA\(_2\) activity of the purified 85-kDa iPLA\(_2\) protein (8). This has been taken to suggest that the active form of iPLA\(_2\) is an oligomer of 85-kDa subunits and that the subunits may associate with each other via their ankyrin repeat regions (8) because such ankyrin repeats participate in many other protein-protein interactions (53). Consistent with this possibility is the observation that iPLA\(_2\) deletion mutants lacking the ankyrin repeat domain but retaining the catalytic domain are catalytically inactive (8). In the long isoform of human iPLA\(_2\), the last iPLA\(_2\) ankyrin repeat is interrupted by a proline-rich insert with some similarities to the Smad4 domain that mediates interactions with signaling partners (43). This raises the possibility that the proline-rich insert in human iPLA\(_2\) might allow it to interact with proteins not recognized by the short isoform of iPLA\(_2\).

That the long isoform of human iPLA\(_2\) can form heterooligomers with altered catalytic properties is suggested by the finding that activity of this protein is reduced when it is co-expressed with truncated iPLA\(_2\)-like proteins that retain the ankyrin repeat domain but lack the catalytic domain (21). These truncated iPLA\(_2\) variants arise from alternatively spliced transcripts that contain intron sequences that result in a premature stop codon, and these transcripts are expressed in human lymphoma cell lines (21). These lymphoma cell lines do not express mRNA species encoding the catalytically active short isoform of iPLA\(_2\) observed in human islets that arises from another mechanism of alternative splicing of the transcript from the iPLA\(_2\) gene. Human islets do not express mRNA species encoding the truncated iPLA\(_2\) variants observed in human lymphoma cells. In contrast, human U937 promonocytic cells express mRNA species encoding both the long and short isoforms of catalytically active iPLA\(_2\) and also express mRNA species encoding an iPLA\(_2\) truncation variant. This indicates that there is heterogeneity among human cells in expression of iPLA\(_2\) gene products that arise from alternative splicing.

The presence of two distinct domains that might mediate protein-protein interactions in the long isoform of human iPLA\(_2\) could cause it to interact with a variety of other proteins. Various participants in hetero-oligomeric complexes with iPLA\(_2\) have been suggested to alter iPLA\(_2\) catalytic properties (21, 47, 61–64). These include calmodulin (63), which physically interacts with and negatively modulates the activity of recombinant 85-kDa iPLA\(_2\) cloned from Chinese hamster ovary cells (64) and rat islets (47). This has been offered as one explanation of why Ca\(^{2+}\) store depletion activates iPLA\(_2\) (65). Although the mechanism underlying this effect may be complex, the effect itself has occurred in vascular myocytes (64), pancreatic islet beta cells (47), and human granulocytes (17). Ca\(^{2+}\) store depletion also activates hydrolysis of arachidonate from phospholipids in differentiated human U937 promonocytic cells by a mechanism that does not require a rise in cytosolic [Ca\(^{2+}\)] (31). Our demonstration that U937 cells express mRNA species encoding iPLA\(_2\) isoforms suggest that iPLA\(_2\) is one candidate for mediating Ca\(^{2+}\) store depletion-induced arachidonate release in those cells. The differences in products of the iPLA\(_2\) gene expressed in specific human cells suggest that iPLA\(_2\) regulation might be complex, as also indicated by the fact that stimuli that induce iPLA\(_2\)-catalyzed arachidonate release and leukotriene production in granulocytes fail to induce these events in lymphocytes, even though both classes of cells express iPLA\(_2\) and leukotriene biosynthetic enzymes (17, 21).

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