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# Cloning and Recombinant Expression of Human Group IIF-Secreted Phospholipase $A_2$

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**Mammalian-secreted phospholipases $A_2$ ($sPLA_2$) form a diverse family of at least nine enzymes that hydrolyze phospholipids to release free fatty acids and lysophospholipids. We report here the cloning and characterization of human group IIF $sPLA_2$ (hGIIF $sPLA_2$). The full-length cDNA codes for a signal peptide of 20 amino acid followed by a mature protein of 148 amino acids containing all of the structural features of catalytically active group II $sPLA_2$s. hGIIF $sPLA_2$ gene is located on chromosome 1 and lies within a $sPLA_2$ gene cluster of about 300 kbp that also contains the genes for group IIA, IIC, IID, IIE, and V $sPLA_2$s. In adult tissues, hGIIF is highly expressed in placenta, testis, thymus, liver, and kidney. Finally, recombinant expression of hGIIF $sPLA_2$ in *Escherichia coli* shows that the enzyme is $Ca^{2+}$-dependent, maximally active at pH 7–8, and hydrolyzes phosphatidylglycerol versus phosphatidylcholine with a 15-fold preference.** 

***Key Words:*** **secreted phospholipase $A_2$; inflammation; cloning; arachidonic acid.**

Phospholipases $A_2$ ($PLA_2$, EC 3.1.1.4) form a superfamily of enzymes that catalyze the hydrolysis of glycerophospholipids at the *sn*-2 position, producing free fatty acids and lysophospholipids (1–4). Many intracellular and secreted phospholipases $A_2$ ($sPLA_2$s) have been cloned in recent years (2, 5), and several of them are involved in a variety of physiological and pathological functions including lipid digestion, cell proliferation, production of lipid mediators of inflammation, antibacterial defense, and cancer (4, 6).

Within the phospholipase $A_2$ superfamily, $sPLA_2$s form a relatively homogenous family of enzymes, and they are characterized by the presence of several disulfides, an overall conserved three-dimensional structure and a common $Ca^{2+}$-dependent catalytic mechanism. Five novel mouse $sPLA_2$s have been cloned during the last three years (7, 8), and the mouse $sPLA_2$ family now comprises 8 distinct 14-16 kDa $sPLA_2$s called group IB, IIA, IIC, IID, IIE, IIF, V, and X, as well as otoconin-95, a $sPLA_2$-like protein with peculiar structural properties (9, 10). Interestingly, genes for group IIA, IIC, IID, IIE, IIF, and V $sPLA_2$s all map to mouse chromosome 4, suggesting the existence of a $sPLA_2$ gene cluster on this chromosome (8). Group IB, IIA, IID, IIE, V, and X $sPLA_2$s, but not group IIF have been cloned from humans (11–13). On the other hand, group IIC $sPLA_2$ appears as a pseudogene in humans (14). In addition, a novel human $sPLA_2$ with a predicted molecular mass of 55 kDa and a central domain similar to insect group III $sPLA_2$s has recently been cloned (15), but it remains to be determined if this $sPLA_2$ is functional in the mouse species. Finally, another structurally distinct human $sPLA_2$ has very recently been cloned (16). All mouse and human $sPLA_2$s have distinct tissue distributions, suggesting that each of them exert non redundant functions that could be related to their different enzymatic properties (6, 17, 18), and/or their binding properties to specific receptors (18–20).

In this paper, we report the cloning, tissue distribution and recombinant expression in *Escherichia coli* of human group IIF (hGIIF) $sPLA_2$. Furthermore, using

The nucleotide sequence reported in this paper has been submitted to the GenBank/EBI Data Bank with Accession No. AF306566.

Abbreviations used: $sPLA_2$, secreted phospholipase $A_2$. A comprehensive abbreviation system for the various $sPLA_2$s is used: each $sPLA_2$ is abbreviated with a lowercase letter indicating the $sPLA_2$ species (m, h, for mouse and human, respectively) followed by capital characters identifying the $sPLA_2$ group (GI, GII, GIII, GV, and GX) and subgroup (A, B, C, D, E, F). kbp, kilobase pair. POPC/PG/PS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine/phosphoglycerol/phosphoserine.

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sequences generated by the Human Genome Project, we show that the gene for hGIIF $sPLA_2$ colocalizes with those for group IIA, IIC, IID, IIE, and V $sPLA_2$s and that these six genes are in fact physically mapped within a region of about 300 kbp of human chromosome 1p.

## MATERIALS AND METHODS

*Molecular cloning of hGIIF $sPLA_2$.* Searching for $sPLA_2$ homologs in gene databases stored at the National Center for Biotechnology using the tBLASTn sequence alignment program (21) resulted in the identification of a human genomic sequence (PAC Clone dJ169023, GenBank Accession No. AL158172) of 142,849 nucleotides containing several regions of homology with mouse group IIF $sPLA_2$. A set of oligonucleotides was designed from this genomic sequence (sense primer 5′-ATGAAGAAGTTCTTCACCGTGGCCA-3′ and reverse primer 5′-ACCCTCCTCCCGCTCTCTCTCTCAAA-3′) and used in RT-PCR experiments on different human cDNAs. A PCR product of the expected size was amplified from spleen, heart, and fetal lung cDNAs. Sequencing of the DNA fragments revealed complete identity with the genomic sequence after its appropriate splicing according to consensus exon–intron boundaries (22).

*Tissue distribution of human $sPLA_2$s.* Multiple tissue cDNA panels (Clontech, Catalog No. K1420-1 and K1421-1) were used as templates in RT-PCR experiments using primers specific for the various human $sPLA_2$s. PCRs were analyzed by agarose gel electrophoresis, transferred to positively charged nylon membranes, and hybridized with specific $^{32}$P-labeled internal oligonucleotide probes.

*Recombinant expression of hGIIF $sPLA_2$.* The preparation of a truncated GST hGIIF sPLA2 construct, bacterial induction and preparation of sulfonated protein from inclusion bodies were carried out as previously described for mouse group IID sPLA2 (7). The hGIIF fusion protein was refolded by a rapid dilution method as follows. Sulfonated protein was dissolved to 10 mg/ml in 4 ml of 6 M guanidine–HCl, 50 mM Tris–HCl, pH 8.0, and added dropwise (~1 drop per second) to 2 liters of refolding buffer (50 mM Tris–HCl, pH 8.0, 0.9 M guanidine–HCl, 10 mM $CaCl_2$, 5 mM freshly added cysteine, 30% acetonitrile) with constant stirring at room temperature. Stirring was continued for a few minutes, and then the solution was allowed to sit without stirring at room temperature for ~2–3 days. The sPLA2 enzymatic activity was monitored with the fluorimetric assay (17) until the activity increase starts to level off. After addition of 5 mM lauryl sulfobetaine (dodecyldimethyl-3-sulfopropylammonium, inner salt) and 1 mM methionine, the protein solution was concentrated by ultrafiltration to 50 ml with a YM-10 membrane (Amicon) and dialyzed 3 times against cleavage buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM $CaCl_2$). Freshly made TPCK-treated trypsin (Sigma) was then added to a final concentration of 0.15 $\mu$g/ml, and the mixture was incubated overnight at room temperature, leading to a ~200-fold increase in $sPLA_2$ activity. The reaction mixture was directly loaded at 3 ml/min on a Vydac 218 TP1010 C18 reverse-phase column equilibrated with solvent A (20% acetonitrile, 0.1% trifluoroacetic acid, 1 mM methionine). Elution was performed at 3 ml/min using a linear gradient (0–6.3% B over 2 min, followed by 6.3–27.5% B over 42 min) of solvent B (100% acetonitrile, 0.1% trifluoroacetic acid, 1 mM methionine). HPLC-purified hGIIF $sPLA_2$ was neutralized with 2 M Tris base, 5 mM lauryl sulfobetaine was added and the sample was concentrated in a Centriprep-10 (Amicon). The protein was then dialyzed against 10 mM Tris, pH 8.0, 0.1 mM DTT, at 4°C for 1 cycle to cleave the disulfide between the cysteine in the C-terminal extension of the hGIIF $sPLA_2$ and free cysteine from the refolding buffer, and then against 10 mM Tris–HCl, pH 8.0 for two cycles. The approximate yield of final product per liter of *E. coli* culture is 3.7 mg. Concentrations of recombinant hGIIF $sPLA_2$ were determined by OD at 280 nm using an extinction coefficient of 10.37 calculated from the amino acid sequence.

*$sPLA_2$ catalytic activity studies.* The $Ca^{2+}$ and pH dependencies of hGIIF $sPLA_2$ were measured with POPC vesicles containing 1-palmitoyl-2-[1-$^3$H]palmitoyl-*sn*-glycero-3-phosphocholine vesicles and POPG vesicles containing 1-palmitoyl-2-[1-$^3$H]palmitoyl-*sn*-glycero-3-phosphoglycerol, respectively (7). Substrate specificity studies were carried out using a slightly modified assay with the fatty acid binding protein (7). Reaction mixtures contained 30 $\mu$M POPC, POPG, or POPS large unilamellar vesicles [0.1 $\mu$m, prepared by extrusion as described (23)] in Hanks' balanced salt solution with 1 mM $Ca^{2+}$, 1 mM $Mg^{2+}$, 9.7 $\mu$g fatty acid binding protein, and 1 $\mu$M 11-dansyl-undecanoic acid at 37°C. Assays were calibrated by adding a known amount of oleic acid to the complete assay in the absence of enzyme.

## RESULTS AND DISCUSSION

### *Cloning of Human Group IIF $sPLA_2$*

Screening of nucleic sequence databases with various mouse $sPLA_2$s led to the identification of a large human genomic fragment of 142849 nucleotides with several regions of homology to mouse group IIF (mGIIF) $sPLA_2$ (8). It was thus likely that this genomic clone contains a complete gene with several exons and introns coding for human group IIF $sPLA_2$. Based on the homology with mGIIF, a set of sense and antisense primers was designed from the genomic sequence to amplify the full-length hGIIF $sPLA_2$ cDNA by RT-PCR. Human cDNAs from spleen, heart and fetal lung were used, and a strong amplification was obtained with spleen cDNA. The sequences of the amplified DNA fragments were found to contain an open reading frame of 168 amino acids comprising a signal peptide of 20 residues followed by a mature protein sequence of 148 residues (Fig. 1). This sequence is 74% identical to mGIIF $sPLA_2$ and contains all of the structural features of mGIIF, including the very long C-terminal extension of 23 amino acids (8). Together, these results indicate that the 168 amino acid sequence corresponds to hGIIF $sPLA_2$ (Fig. 1).

The hGIIF mature protein sequence (calculated molecular mass 15,598 Da) is the most acidic $sPLA_2$ identified so far in mammals, with a calculated p*I* of 4.51. The 23 amino acid C-terminal extension of hGIIF also appears relatively acidic, as it contains 3 glutamic acid residues and no basic residues. Furthermore, one-third (8 of 23) of the residues of this C-terminal extension are proline residues. Interestingly, these specific features appear to be conserved among species, as the mouse group IIF C-terminal sequence is also acidic and proline-rich. The odd cysteine residue found in the mGIIF $sPLA_2$ C-terminal extension is also conserved in the hGIIF $sPLA_2$ sequence. The possible involvement of these amino acids in the putative homo- or heterodimerization of group IIF $sPLA_2$s remains to be determined. Four potential N-glycosylation sites were found in the mature sequence of hGIIF $sPLA_2$ at positions 79, 89, 110, and 134 (Fig. 1) and only three of

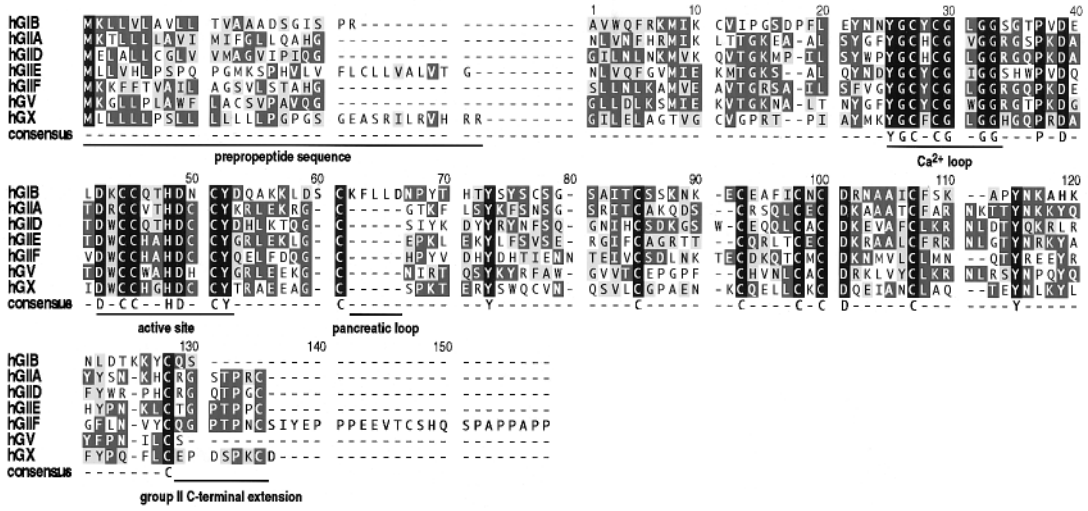


**FIG. 1.** Alignment of the amino acid sequences of human $sPLA_2$s. Sequences of full-length $sPLA_2$ proteins are shown. A consensus sequence for the 7 group I/II/V/X human $sPLA_2$s is presented.

them (positions 79, 89, and 134) are conserved in the mGIIF sequence (8).

An alignment of the amino acid sequences of the 7 human catalytically active group I/II/V/X $sPLA_2$ collection is presented in Fig. 1, and their respective levels of identity is shown in Table 1. hGIIF $sPLA_2$ contains the different residues which are conserved in all catalytically active $sPLA_2$s and is particularly well-conserved with other human $sPLA_2$s in the $Ca^{2+}$-loop and active site domains. hGIIF $sPLA_2$ however shows low levels of identity with other human $sPLA_2$s, and the most closely related $sPLA_2$ is hGIID with only 41% identity (Table 1), indicating that hGIIF $sPLA_2$ is not an isoform of the previously cloned human $sPLA_2$s. It should be noted that the highest level of identity between any two $sPLA_2$s is observed between GIIA and GIIE [55% of identity in human species (Table 1) and 51% in mouse species (8)].

### *hGIIF $sPLA_2$ Gene Maps to Chromosome 1 and Belongs to a $sPLA_2$ Gene Cluster*

We have previously reported that the six genes for mGIIA, mGIIC, mGIID, mGIIE, mGIIF, and mGV

**TABLE 1**

Level of Amino Acid Sequence Identity (%) between the Different Human $sPLA_2$s

| $sPLA_2$ | hGIIA | hGIID | hGIIE | hGIIF | hGV | hGX |
|---|---|---|---|---|---|---|
| hGIB | 35 | 36 | 35 | 27 | 30 | 29 |
| hGIIA | | 50 | 55 | 33 | 44 | 35 |
| hGIID | | | 39 | 41 | 42 | 39 |
| hGIIE | | | | 35 | 41 | 38 |
| hGIIF | | | | | 33 | 29 |
| hGV | | | | | | 37 |

$sPLA_2$s are located in the distal part of mouse chromosome 4 and most likely form a $sPLA_2$ gene cluster (8). Furthermore, the genes for hGIIA, hGIIC, and hGV $sPLA_2$s have also been proposed to form a gene cluster positioned between the genetic markers AFM217zc3 and AFM290vb9 (14). Here we have taken advantage of the human genome sequencing project to show that the 6 corresponding human $sPLA_2$ genes are in fact located very close to each other within a DNA fragment of about 300 kbp. The organization of the $sPLA_2$ gene cluster is presented in Fig. 2.

The human PAC Clone dJ169023 (GenBank No. AL158172) of 141,865 bp that contains the *hGIIF* gene was generated by the sequencing program of human chromosome 1, assigning the *hGIIF* gene to this chromosome. In addition to the *hGIIF* gene, this PAC clone contains also the full-length genes for *hGV*, *hGIID*, as well as the *hGIIC* pseudogene. The *hGIIA* and *hGIIE* genes were found to be localized on the overlapping PAC clone dJ169023 (GenBank No. AL358253) in the telomeric direction (Fig. 2). At present, the available sequence of this PAC clone is composed of 49 unordered contigs of different lengths. Based on alignments of these different contigs with the sequence of the PAC clone AL158172, the overlapping sequence between AL158172 and AL358253 is estimated to be about 28 kbp. The relative orientation of *hGIIA* and *hGIIE* with the other $sPLA_2$ genes and the exact distances between *hGIIA* and *hGIIE* genes, and *hGIIA* and *hGV* genes are unknown. However, based on the full-length sequence of the mouse cosmid clone of 41,125 bp (GenBank No. AC002108) that contains the *mGIIA* gene and a portion of the *mGV* gene (24), it is likely that the *hGIIA* and *hGV* genes are organized in a head to tail orientation and that the *hGIIE* gene is localized in the telomeric direction, as presented in Fig. 2.

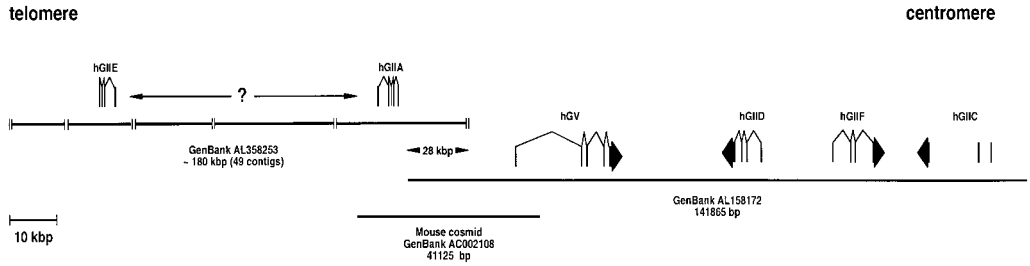


**FIG. 2.** Schematic diagram of the organization of the human chromosome 1p35 $sPLA_2$ gene cluster. The total length between *hGIIE* gene and *hGIIC* pseudogene is about 300 kbp. The PAC clone GenBank No. AL358253 is not yet fully sequenced and the hatched bars indicate the different contigs of this PAC clone. The orientation and exon–intron boundaries of the different $sPLA_2$ genes are schematically shown. The possible presence of 5′ noncoding exons in the *hGIIC, hGIID, hGIIE* and *hGIIF* genes remains to be determined. The orientation and exact positions of the *hGIIE* and *hGIIA* genes are unknown. However, based on the sequence of the mouse cosmid KH1 (GenBank No. AC002108) that contains the *mGIIA* gene and a portion of the *mGV* gene, it is likely that the *hGIIA* and *hGV* genes are in a head to tail orientation and that the *hGIIE* gene is localized closer to the telomere.

### *Analysis of the tissue distribution of hGIIF $sPLA_2$*

The tissue distribution of hGIIF and other human $sPLA_2$s was analyzed by RT-PCR experiments using commercial human cDNA panels. As shown in Fig. 3, hGIIF $sPLA_2$ is expressed at high levels in placenta, testis, thymus, and at lower levels in heart, kidney, liver, and prostate. Very low signals are observed in skeletal muscle, pancreas, small intestine, and spleen. In the mouse species, mGIIF transcripts were detected mostly in testis but also in several other tissues (8). In the future, it will be interesting to analyze the expression of hGIIF $sPLA_2$ in embryos, since high levels of mGIIF transcripts were observed at different stages of embryonic development (8). The different patterns of expression of human $sPLA_2$s presented in Fig. 3 and that previously found for hGIIE $sPLA_2$ (11) clearly indicate that all human $sPLA_2$s including hGIIF most probably have nonredundant functions. Overall, the tissue distributions of the human $sPLA_2$s resemble those previously found by Northern blot analysis (12, 13, 15). Finally, it is interesting to note that all human $sPLA_2$s are expressed in pancreas and that placenta, lung, colon and small intestine are also rich sources of $sPLA_2$.

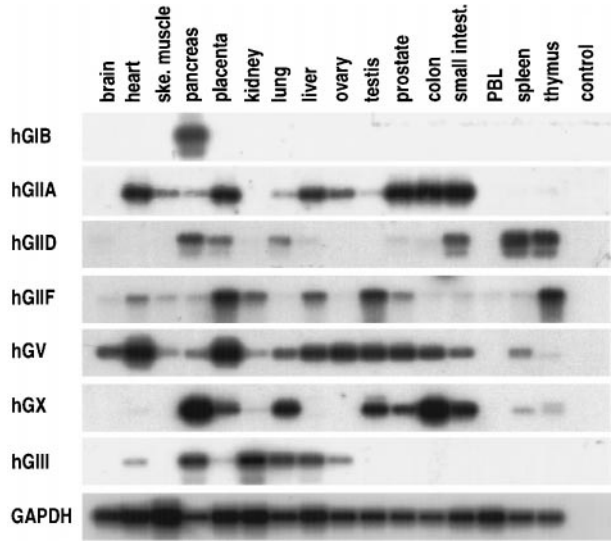


**FIG. 3.** Tissue distribution of the human $sPLA_2$s, as determined by RT-PCR on human adult cDNA panels. The amplified products were analyzed by Southern blot as described under Materials and Methods. No amplification was observed when cDNA was omitted in the PCR (control lane).

### *Recombinant Expression of hGIIF $sPLA_2$*

To study the interfacial kinetic properties of hGIIF $sPLA_2$, we produced this enzyme as a recombinant fusion protein in *E. coli.* Inclusion bodies containing hGIIF fusion protein were solubilized and reduced, and free cysteines were sulfonated. Rapid dilution of the sulfonated protein into a buffer containing 30% acetonitrile, to minimize protein aggregation, produced refolded fusion protein which displayed maximal activity after 2–3 days. The fusion protein contains a factor Xa recognition site adjacent to the N-terminal residue of mature hGIIF which could be efficiently cleaved by using Factor Xa and or trypsin. Cleaved hGIIF $sPLA_2$ was purified to homogeneity by chromatography on a C18 reverse phase column, and the overall yield of purified hGIIF $sPLA_2$ is 3.7 mg per liter of *E. coli* culture (data not shown).

The interfacial enzymatic properties of hGIIF $sPLA_2$ are summarized in Fig. 4. The hydrolysis of phospholipid vesicles by hGIIF $sPLA_2$ is strictly $Ca^{2+}$-dependent, as expected for a typical $sPLA_2$. Using PC vesicles as substrate, the enzyme displayed a hyperbolic dependence on the concentration of $Ca^{2+}$ (Fig. 4A), and an apparent $KCa^{2+}$ of 140 ± 40 $\mu$M was calculated. Figure 4B shows that the rate of hydrolysis of phosphatidylglycerol vesicles by hGIIF $sPLA_2$ increases with pH in the range 5–7, as expected from the deprotonation of the active site histidine residue, and then decreases slightly at pH above 7. The relative rates for the hydrolysis of POPG,

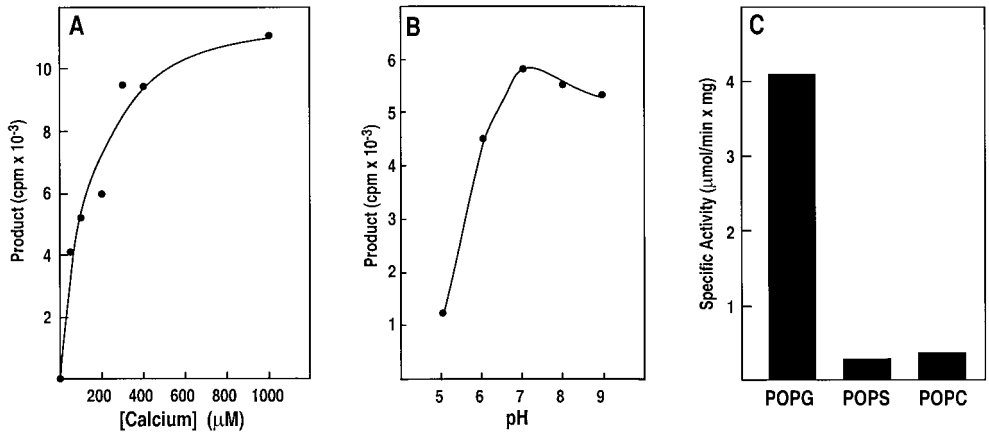


**FIG. 4.** Enzymatic properties of recombinant hGIIF $sPLA_2$. (A) $Ca^{2+}$-dependence of the hydrolysis of phosphatidylcholine vesicles; (B) pH dependence of the hydrolysis of phosphatidylglycerol vesicles; (C) initial velocities for the hydrolysis of the indicated phospholipid vesicles. Full experimental details are provided under Materials and Methods.

POPS, and POPC vesicles by hGIIF $sPLA_2$ are compared in Fig. 4C. As for all mammalian $sPLA_2$s examined so far (7, 8), the enzymatic activity of hGIIF $sPLA_2$ is highest with anionic POPG vesicles, which probably reflects the relatively high affinity of all $sPLA_2$s for POPG vesicles. Although hGIIF hydrolyses POPC at only ~6% of the rate of POPG, this enzyme is much more active on POPC vesicles than hGIIA $sPLA_2$, which displays a greater than 105-fold preference for POPG versus POPC vesicles (8). In this context, hGIIF appears more similar to hGV and hGX $sPLA_2$s, which are 3- and 10-fold more active on POPG versus POPC vesicles, respectively (17). Whether exogenous hGIIF $sPLA_2$, like hGX $sPLA_2$, is able to efficiently release arachidonic acid from adherent cells will be interesting to analyze in the future (17).

In conclusion, we have reported here the cloning and recombinant expression of hGIIF $sPLA_2$. This group II $sPLA_2$ has unique structural features including a long, proline-rich C-terminal extension with an odd cysteine, and a very low pI value. It also has a specific tissue distribution and a fairly high propensity to hydrolyze POPC versus POPG compared to the other $sPLA_2$s. Furthermore, the gene for hGIIF $sPLA_2$ maps to chromosome 1 together with 5 other $sPLA_2$ genes to form a $sPLA_2$ gene cluster that spans about 300 kbp. Interestingly, 5 of these 6 genes code for group II enzymes and share relatively high levels of identity (Table 1). The last gene coding for group V $sPLA_2$ is in fact also related to group II $sPLA_2$ genes, as group V $sPLA_2$ does not contain a propeptide sequence and displays higher levels of identity to group II $sPLA_2$s than to group IB and X $sPLA_2$s (Table 1). It is thus likely that these 6 different genes have arisen from recent and successive gene duplication events. It should be also noted that group IIA, IIC, IID, IIE, and V $sPLA_2$s are all basic enzymes while group IIF is very acidic. On the other hand, group IB and X $sPLA_2$s appear more divergent in sequence and are located on different chromosomes (13). Both contain a propeptide sequence and the group I specific disulfide bond between cysteines 11 and 77. Whether group IB, X or one of the group II-like $sPLA_2$s is more related to the $sPLA_2$ ancestor gene of the group I/II/V/X $sPLA_2$ collection (5) remains however to be determined.

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