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Analysis of expression of secreted phospholipases A_2 in mouse tissues at protein and mRNA levels

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Abstract

Secreted phospholipases A_2 (sPLA$_2$) form a group of low-molecular weight enzymes that catalyze the hydrolysis of phospholipids. Some sPLA$_2$s are likely to play a role in inflammation, cancer, and as antibacterial enzymes in innate immunity. We developed specific and sensitive time-resolved fluroimmunoassays (TR-FIA) for mouse group (G) IB, GIIA, GIID, GIIE, GIIF, GV and GX sPLA$_2$s and measured their concentrations in mouse serum and tissues obtained from both Balb/c and C57BL/6J mice. We also analyzed the mRNA expression of the sPLA$_2$s by quantitative real-time reverse transcriptase PCR (qPCR). In most tissues, the concentrations of sPLA$_2$ proteins corresponded to the expression of sPLA$_2$s at the mRNA level. With a few exceptions, the sPLA$_2$ proteins were found in the gastrointestinal tract. The qPCR results showed that GIB sPLA$_2$ is synthesized widely in the gastrointestinal tract, including esophagus and colon, in addition to stomach and pancreas. Our results also suggest that the loss of GIIA sPLA$_2$ in the intestine of GIIA sPLA$_2$-deficient C57BL/6J mice is not compensated by other sPLA$_2$s under normal conditions. Outside the gastrointestinal tract, sPLA$_2$s were expressed occasionally in a number of tissues. The TR-FIAs developed in the current study may serve as useful tools to measure the levels of sPLA$_2$ proteins in mouse serum and tissues in various experimental settings.

Keywords: Mouse; mRNA; Protein concentration; Secreted phospholipase A_2; Time-resolved fluoroimmunoassay; Tissue

1. Introduction

Phospholipases A_2 (PLA$_2$) are enzymes that catalyze the hydrolysis of the ester bond at the *sn*-2 position of phospholipids to generate free fatty acids and lysophospholipids [1–3]. Over the past few years, it has been realized that PLA$_2$s constitute a superfamily of enzymes comprising a set of at least ten distinct intracellular enzymes [4–6] and another set of up to 12 different extracellular [7–11], i.e., secreted PLA$_2$s (sPLA$_2$)[1]. The intracellular PLA$_2$s are involved in the production of potent lipid mediators and in phospholipid homeostasis in a variety of physiological and pathological settings [1,12–15]. The specific biological functions of different sPLA$_2$s are still largely unknown [9–12,16,17]. However, based on a number of previous studies, it can be assumed that the functions of sPLA$_2$s are highly diverse and range from the release of lipid mediators to host defense and vertebrate embryonic development [18]. For

Abbreviations: AS, analytical sensitivity; BSA, bovine serum albumin; DTPA, diethylenetriaminepentaacetic acid; cps, counts per second; cv, coefficient of variation; G, group; IgG, Immunoglobulin G; LPS, lipopolysaccaride; PBS, phosphate buffered saline; PLA$_2$, phospholipase A_2; qPCR, quantitative real-time RT-PCR; RT-PCR, reverse transcriptase polymerase chain reaction; S.D., standard deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; S.E.M., standard error of the mean; sPLA$_2$, secreted phospholipase A_2; TR-FIA, time-resolved fluoroimmunoassay; TSA, Tris–HCl, saline, azide buffer

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[1] A comprehensive abbreviation system for the various mammalian sPLA$_2$s is used: each sPLA$_2$ is abbreviated with a lowercase letter indicating the sPLA$_2$ species (m and h for mouse and human, respectively), followed by uppercase letters identifying the sPLA$_2$ group (GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GIX, GX, GXI, and GXII).

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instance, sPLA$_2$s are involved in the release of lipid mediators in various settings, including inflammation and associated diseases [11,19,20] and in various types of cancer [21–24]. sPLA$_2$s are also involved in lipid digestion and obesity [25,26], activation of different types of immune cells [27,28], lipoprotein metabolism and atherosclerosis [20,29,30], hydrolysis of lung surfactant, acute respiratory distress syndrome [31], skin barrier homeostasis [11,32,33], and host defence against bacteria [34–38] and possibly against viruses [39,40] and parasites [41].

The different members of the sPLA$_2$ family have no sequence homology with the intracellular PLA$_2$s, but share a number of common properties including a relatively low molecular mass of 14 to 19 kDa, numerous intramolecular disulfide bridges, and a Ca^{2+}-dependent catalytic mechanism. sPLA$_2$s fall into three main structural collections [9]. GIB, GIIA, GIIC, GIID, GIIE, GIIF, GV and GX sPLA$_2$s and otoconin-95 belong to the I/II/V/X PLA$_2$ collection, while GIII and GXII sPLA$_2$s are members of the group III and XII collections, respectively. GIB, GIIA, GIIC, GIID, GIIE, GIIF, GIII, GV, GX and GXIIA sPLA$_2$s are catalytically active enzymes, while otoconin-95 and GXIIB sPLA$_2$ are sPLA$_2$-like proteins that naturally lack enzymatic activity because of mutations in the catalytic site. Clearly, the different mammalian sPLA$_2$s are not isoforms as their sequence identities are only around 15% [8,9], they have distinct enzymatic properties [42], and show different tissue distribution patterns in both mice and humans. Consequently, in various tissues, the different sPLA$_2$s may exert distinct biological functions that may be dependent or independent of their enzymatic activities [8,17,43].

The group IB and IIA sPLA$_2$s are the only mammalian sPLA$_2$s that have been cloned and characterized after the initial purification of the native proteins from various mammalian species [25,44–49]. All the other sPLA$_2$s have been cloned by sequence homology [50] and *in silico* DNA database searching [8,9,51–54]. As a result, most of our current knowledge on the expression of sPLA$_2$s in mammalian tissues is derived from the analysis of expression at the mRNA level. In mouse, only the GIIA sPLA$_2$ has been purified from tissue extracts and then cloned [45], and its expression analyzed in various tissues at both mRNA and protein levels [32,55–58]. mRNA for mouse pancreatic-type GIB sPLA$_2$ was found in abundance in stomach and pancreas and, at lower levels, in non-digestive tissues including lung, liver and spleen [56,59,60]. mRNA for mouse GIIC sPLA$_2$ was detected in testis and pancreas [56,61]. mRNA for mouse GIID sPLA$_2$ was found at relatively low levels in several tissues including spleen, thymus, pancreas, lung and skin [56,62]. mRNA for mouse GIIE sPLA$_2$ was detected in uterus, testis, thymus, small intestine, lung and spleen [52,56]. mRNA for mouse GIIF sPLA$_2$ was found in testis, small intestine, pancreas, eye and brain [56]. mRNA for mouse GV sPLA$_2$ was detected in heart, lung, spleen and testis [56,63]. mRNA for mouse GX sPLA$_2$ was detected in testis and stomach among other tissues [56,64]. Finally, mRNA for the most recently cloned mouse GXIIA and GXIIB sPLA$_2$s were found in various tissues including small intestine, liver and pancreas. Of note, inflammatory stimuli caused up-regulation or down-regulation of the expression of mRNA for various mouse sPLA$_2$s as detected by northern blotting or RT-PCR [51,52,63,65–68]. Besides GIB and GIIA sPLA$_2$s, the tissue distribution pattern of the different human sPLA$_2$s has also been mainly determined by analyzing the expression at the mRNA level [8,9,11,17,29].

Time-resolved fluorometry allows the use of non-radioactive molecules including lanthanides, such as Europium (Eu), for labeling immunoglobulins for immunoassays. The advantages of fluorescence emitted by lanthanides are large Stoke's shift (separation of excitation and emission wavelengths) and long decay time. The latter allows the measurement of fluorescence after a short delay time during which the short-lived background fluorescence emitted by sample macromolecules such as nucleic acids and proteins disappears, which increases the sensitivity of the method [69]. Time-resolved fluoroimmunoassays (TR-FIA) have been developed earlier for human GIB and GIIA sPLA$_2$s [70,71] and recently for the complete set of human sPLA$_2$s [72] for measuring the concentrations of these proteins in serum.

As reviewed above, the expression of sPLA$_2$s has been extensively studied at the mRNA level in numerous mouse and human tissues. However, no quantitative data on sPLA$_2$ expression has been published at the protein level. The purpose of the current study was to develop TR-FIAs for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX sPLA$_2$s and to investigate their protein levels in mouse serum and a collection of adult mouse tissues. In mouse tissues, the results were also compared to the expression of sPLAs at the mRNA level.

2. Materials and methods

2.1. Production of recombinant mouse sPLA$_2$s and antibodies

Recombinant mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX sPLA$_2$s were produced as described for human sPLA$_2$s [42], and shown to be pure and fully folded by SDS-PAGE and mass spectrometry analyses [42]. Polyclonal rabbit antisera against each mouse sPLA$_2$s were produced by using recombinant sPLA$_2$s as antigens as described for the antisera to human recombinant sPLA$_2$s [73]. The antiserum for each mouse sPLA$_2$ is capable of detecting ~1 ng of sPLA$_2$ by immunoblot analysis. Each antiserum is specific in that each one does not detect 50 ng of each of the other sPLA$_2$s when analyzed by immunoblotting [73].

2.2. Purification of antibodies from rabbit antisera

IgG was isolated from rabbit anti-sPLA$_2$ antisera by protein A-affinity chromatography. One ml of antiserum was diluted with 9 ml of phosphate buffered saline (PBS, 20 mM sodium phosphate buffer, pH 7.0, containing 0.9% NaCl), passed through a 0.2-μm filter (Gelman Sciences, Ann Arbor, USA) and applied to a 1-ml HiTrap Protein A HP-column (Amersham Biosciences, Uppsala, Sweden) at a flow rate of 1 ml/min. The eluent was monitored for absorbance at 280 nm. The column was washed with PBS until absorbance at 280 nm reached the baseline and thereafter eluted with glycine buffer (0.1 M glycine–HCl, pH 2.7). Chromatography equipment: 2132 Microperpex Peristaltic pump, 2238 Uvicord S II UV-detector, 2210 Recorder and 2212 Helirac fraction collector, all from LKB Bromma, Sweden. For neutralization, the IgG fraction was dialyzed against PBS overnight at 4 °C and the protein concentration was determined spectrophotometrically by measuring absorbance at 280 nm (PU8740 UV/VIS scanning spectrophotometer, Philips, Cambridge, England).

2.3. Labeling of anti-mouse-PLA$_2$ antibodies

IgG purified by protein A was concentrated by lyophilization and 1–2 mg of IgG was dissolved in 300 μl of water and combined with 0.2 mg of Delfia

Eu-labeling reagent (Perkin Elmer Wallac, Turku, Finland) dissolved in 100 μl of water. The pH of the solution was raised to 9–10 by adding 20 μl of 1 M Na_2CO_3, and the labeling reaction was allowed to proceed overnight at 4 °C. The labeled IgG was separated from unreacted labeling reagent by size exclusion chromatography on a 1.6-cm diameter column containing a combination of 16 cm of Superdex 75 gel (Pharmacia, Uppsala, Sweden) layered on 40 cm of Trisacryl GF 2000 gel (LKB, Bromma, Sweden). The column was equilibrated and eluted with Tris–HCl, saline, azide buffer (TSA, 50 mM Tris–HCl, pH 7.75, containing 0.9% NaCl and 0.05% NaN_3), and the eluent was monitored for absorbance at 280 nm and collected in 1 ml fractions. Time-resolved fluorescence of the high molecular weight IgG-containing fractions was determined by adding 1 μl of fraction to 1 ml of Delfia Enhancement Solution that contains acetic acid, chelators and Triton X-100 (Perkin Elmer, Wallac, Turku, Finland) and by measuring the time-resolved fluorescence (1230 Arcus Fluorometer, Wallac, Turku). The fraction with the highest time-resolved fluorescence value (counts per second, cps) and 8–11 adjacent fractions were pooled and used as stock solution for the Eu-labeled IgG tracer for TR-FIA. The Eu-labeled IgG stock solutions were stored at 4 °C.

2.4. Characterization of the labeled antibodies

The IgG concentration of Eu-labeled stock solution was calculated as follows: mg of protein in labeling reaction x (sum of the cps in pooled high Eu-count IgG fractions/sum of the cps in all IgG fractions). The Eu concentration of labeled antibodies was determined by diluting the Eu-labeled stock solutions in DELFIA Enhancement Solution, measuring the time-resolved fluorescence and comparing the signal to Eu standard curve made from the 1-nM Eu Standard Solution (Perkin Elmer Wallac, Turku, Finland). The Eu/IgG ratio (mol/mol) of the labeled stock solutions was calculated as follows: Eu concentration/protein concentration.

2.5. Coating microtiter wells

Anti-mouse-$sPLA_2$ IgG was immobilized to the microtiter wells (96-well Delfia Microtiter Plates for TR-FIA, Perkin Elmer Wallac, Turku, Finland) by passive adsorption. IgG was first mixed with 3 volumes of acidic solution (125 μl of concentrated HCl in 50 ml of water) by slow manual rotation for 1 min. TSA was added to give an IgG concentration of 10 μg/ml, and the solution was allowed to stand at room temperature for 1 h. Wells were coated with 100 μl of IgG solution overnight at room temperature and washed four times with TR-FIA wash solution (10 mM Tris–HCl, pH 7.75, containing 0.9% NaCl, 0.038% NaN_3 and 0.02% Tween 20) by using plate washer (1296-024 Delfia Platewash, Wallac, Turku, Finland). Unreacted sites were blocked by adding 300 μl of blocking solution containing 0.1% BSA (Sigma Aldrich Inc., St. Louis, MO, USA), 6% D-sorbitol, 3.9% diethylenetriaminepentaacetic acid (DTPA, Merck, Darmstadt, Germany) and 1 mM $CaCl_2$, and overnight incubation at room temperature. The plates were stored in a moistened atmosphere at 4 °C.

2.6. TR-FIA procedure

Prior to use, IgG-coated microtiter wells were washed twice with TR-FIA washing solution. Thereafter, 10 μl of analyte (serum samples, tissue extracts, standard solutions or Delfia Assay Buffer in blank wells) and 90 μl of Delfia Assay Buffer (Tris–HCl buffered NaCl solution, pH 7.8, containing NaN_3, BSA, bovine gamma globulins, Tween 40, DTPA and inert red dye, Perkin Elmer Wallac, Turku, Finland) were added to the wells and incubated at room temperature with shaking at 240 cycles/min for 30 min (1296–001 Delfia Plateshake, Wallac, Turku). Wells were washed four times with TR-FIA washing solution, and 100 μl of Eu-labeled IgG tracer (0.5 μg/ml for GIB $sPLA_2$ and 1 μg/ml for GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s diluted with Delfia Assay Buffer) were added followed by incubation and washing steps as described above. After washing, 100 μl of Delfia Enhancement Solution were added to wells, incubated at room temperature for 5 min with shaking at 240 cycles/min and thereafter for 10 min without shaking, and time-resolved fluorescence was measured (1420 Victor Multilabel Counter, Wallac, Turku).

2.7. Specificity, standard curves, analytical sensitivities, reproducibility and analytical recoveries of the TR-FIAs

To study the specificity of the rabbit antisera, the cross-reactivity of the purified anti-mouse-$sPLA_2$ antibodies was tested against the different mouse $sPLA_2$s. This was done by diluting each of the seven recombinant proteins in Delfia Assay Buffer (400 μg/l for GIIA, GIID, GIIE, GIIF and GV $sPLA_2$ and 200 μg/l for GIB and GX $sPLA_2$) and by measuring each diluted sample with all seven TR-FIAs.

Standard curves were constructed to convert the cps-readings from the fluorometer to the $sPLA_2$ protein concentration in the sample and to study the linear measuring range. For standard curves, recombinant mouse $sPLA_2$s were diluted to appropriate concentrations in Delfia Assay Buffer and measured with the pertinent TR-FIAs.

To study the analytical sensitivities of the TR-FIAs, six replicates of a sample with no analytes present (blanks) were measured with TR-FIAs and the mean and S.D. of the signals were calculated. The analytical sensitivity was determined as the concentration corresponding the cps-value of mean ±3 S.D. of the zero sample.

To study the reproducibility of the TR-FIA, standard samples containing 12.5 and 50 μg/l of each $sPLA_2$ were measured on four subsequent days.

The suitability of TR-FIAs for measuring serum and tissue samples was tested by analytical recovery experiments. The samples for the recovery tests were prepared by adding each recombinant mouse $sPLA_2$s to give calculated concentrations of 50 and 100 μg/l of added $sPLA_2$ to mouse serum and three tissues extracts with different protein contents (liver, rectum and thymus). The concentrations of $sPLA_2$s in the samples before and after the $sPLA_2$ additions were measured with TR-FIAs. Analytical recovery was calculated as follows: (measured increase in concentration / expected increase in concentration) × 100%.

2.8. Tissue samples

To study the distribution of $sPLA_2$s in GIIA $sPLA_2$-expressing Balb/c (+/+) and GIIA $sPLA_2$-deficient C57BL/6J (−/−) mice, serum and tissue samples were obtained from both mouse strains. C57BL/6J mice have a disrupted GIIA $sPLA_2$ gene because of a natural frameshift mutation in exon coding for the active site. Therefore, C57BL/6J mice are deficient for the production of functional endogenous GIIA $sPLA_2$ [74] whereas Balb/c mice have a normal GIIA $sPLA_2$ gene.

Three male C57BL/6J and three male and three female Balb/c mice, 8–10 weeks old, were anesthetized lightly with diethyl ether, and blood samples were collected from the tip of the tail. To isolate serum, the samples were incubated at room temperature for 2 h and centrifuged at 2400×*g* for 15 min at 4 °C. The mice were euthanized by cervical dislocation and tissue samples (approximately 50–100 mg) were taken from salivary gland, oesophagus, glandular stomach, liver, pancreas, small intestine, colon, rectum, brain, aorta, heart, kidney, prostate, testis, urinary bladder, ovary, uterus, lung, adrenal gland, lacrimal gland, muscle, skin, spleen, thymus and white fat. Tissue samples were homogenized with a mechanical homogenizer (Ultra-Turrax T25 homogenizer, IKA, Staufen, Germany) in 400 μl of saline and centrifugated at 4000×*g* for 15 min at 4 °C. The supernatants were recovered and used for TR-FIAs after appropriate dilutions in Delfia Assay Buffer. The experiments were approved by the local ethical committee and performed in accordance with the institutional guidelines.

The concentrations of GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s in serum samples and tissue homogenates were measured with the TR-FIAs developed. For GIB $sPLA_2$ TR-FIA, the homogenates of pancreas and glandular stomach were diluted 100- and 500-fold in Delfia Assay Buffer, respectively, and for GIIA $sPLA_2$ TR-FIA, the homogenates of small intestine were diluted 50-fold. The protein content of the tissue homogenates was determined spectrophotometrically with Multiscan plate reader (Labsystems, Helsinki, Finland) by using Fast and Easy Protein Assay (BioCell Products Oy, Helsinki, Finland) and BSA as standard [75].

2.9. Analysis of tissue distribution of mouse $sPLA_2$ by qPCR

For quantitative real-time RT-PCR (qPCR), tissues from male and female adult Balb/c mice were homogenized with polytron in RNA lysis buffer with antifoam A (#A5758, Sigma Aldrich, St Quentin Fallavier, France). Total RNA

was isolated using the SV total RNA kit including a DNase I treatment (Promega France, Charbonnieres-Les-Bains, France). First-strand cDNA was synthesized from 10 μg of total RNA using MMLV reverse transcriptase (Promega France, Charbonnieres-Les-Bains, France) with oligo(dT)$_{15}$ and random primers. qPCR was carried out in 96-well ABgene plates using the GENEAMP® 5700 Sequence Detection System apparatus (Applied Biosystems, Applera France, Courtaboeuf, France) with the qPCR Master Mix Plus for SYBR® Green I (Eurogentec, Seraing, Belgium). All reactions were performed in a total volume of 16 μl and contained 200 ng of reverse transcribed RNA (based on the initial RNA concentration) and 250 nM of each set of primers. All sets of primers were designed using the Primer Express program from Applied Biosystems and are available on request. The efficiency and specificity of primer sets were validated using serial dilutions of the individual mouse sPLA$_2$ cDNAs subcloned into plasmids and of mixed mouse tissue cDNAs. Negative (no added template) and positive (sPLA$_2$ cDNA plasmid template) controls were run for each set of experiments. Thermal cycles were set at 95 °C for 10 min, followed by 40 cycles comprising each a denaturation step at 95 °C for 0.15 min, and an annealing/extension step at 60 °C for 1 min. Amplification of the appropriate product was verified in all reactions by analyzing the dissociation curves that were obtained after PCR with the following steps: 15 s at 95 °C, 20 s at 60 °C, and then a slow ramp of 20 min from 60 °C to 95 °C. The abundance of the target sPLA$_2$ mRNA was calculated relative to the expression of the 18 S ribosomal RNA that served as a reference gene [76]. The "comparative Ct" method (outlined in ABI prism 7700 Sequence Detection System Bulletin #2) was used to determine the relative quantities of sPLA$_2$ mRNA in different tissues. A relative abundance of 1 corresponded to very low expression levels of sPLA$_2$s with Ct values ≥32 (Ct values for 18 S were between 7 to 12 in parallel experiments).

2.10. Statistics

Non-parametric Mann–Whitney *U* test was used to study the significances of the differences between the concentrations of sPLA$_2$s in tissue homogenates and sera between Balb/c and C57BL/6J mice. Values are expressed as mean ± S.E.M. All statistical calculations were performed with Statistica Software (StatSoft, Tulsa, OK, USA).

3. Results

3.1. Characterization of the labeled antibodies

The Eu/IgG ratios (mol/mol) were calculated from Eu concentration and protein concentrations of labeled antibody stock solutions. The ratios were 10 for GIB sPLA$_2$, 5 (GIIA sPLA$_2$), 13 (GIID sPLA$_2$), 16 (GIIE sPLA$_2$), 12 (GIIF sPLA$_2$), 10 (GV GLA$_2$) and 18 (GX sPLA$_2$). No effort has been made to standardize and optimize fluorescence labeling of the antibodies.

3.2. Specificity, standard curves, analytical sensitivities, reproducibility and analytical recoveries of the TR-FIAs

Specificity of the antisera. To study the specificity of the antibodies used in each sPLA$_2$ TR-FIA, a cross-reactivity test was done. The cross-reactivities were calculated as signal-to-blank (i.e., cps-values divided by mean of the blank cps-values for the respective assay). The absence of cross-reactivity thus gives a value close to 1.0. In the current tests, the ratios for the specific antigen-antibody pairs varied from 25 to 141, whereas the ratios for the non-relevant antibody and antigen combinations ranged from 0.6 to 1.9 indicating practically complete absence of cross-reactivity between the antibodies used in the current TR-FIAs (Table 1).

Table 1
The absence of cross-reactivity between the non-relevant antibodies and antigens

Sample	Assay						
	GIB	GIIA	GIID	GIIE	GIIF	GV	GX
GIB	141	0.8	1.0	1.0	1.0	1.0	1.2
GIIA	1.2	56	0.7	0.9	1.0	1.3	1.1
GIID	1.2	0.8	43	0.7	1.0	0.7	1.1
GIIE	1.1	0.8	1.0	156	1.0	0.7	1.1
GIIF	1.5	0.8	0.9	0.9	172	0.6	1.9
GV	1.5	0.8	0.9	0.9	1.1	25	1.5
GX	0.7	0.8	0.9	0.9	1.0	0.6	32

Each of the seven recombinant sPLA$_2$ antigens was tested with each seven specific TR-FIAs by using the IgGs purified from the respective antisera. The results are expressed as signal-to-blank-ratio (i.e., cps-values divided by the mean blank cps-values for the respective assay). A value close to 1.0 indicates the absence of cross-reactivity.

Standard curves. To convert the cps-readings of the samples to sPLA$_2$ protein concentration values, standard curves were constructed by analyzing different concentrations of analytes, i.e., recombinant sPLAs, with TR-FIAs. Each concentration was analyzed in six replicates and the intra-assay cv% of the measurements forms the precision profile of the assay. The precision profile represents the change in the imprecision of an assay when the measured concentration changes. Standard curves and precision profiles are illustrated in Fig. 1. The signal responses of the TR-FIAs were variable, most probably due to differences in the biological properties of the antibodies and/or labeling. The signal responses of all seven TR-FIAs were high, with that for GIIE sPLA$_2$ having a considerably higher response than the others. All the assays were linear to 200 μg/l and linear regression formulas were used in calculations to convert the cps-readings to sPLA$_2$ concentration values (μg/l).

The analytical sensitivities were calculated from blank measurements by determining the concentration corresponding to mean fluorescence plus three standard deviations (S.D.) of six zero measurements. The analytical sensitivity is the lowest concentration that can be reliably distinguished from background noise. The analytical sensitivities were 1.6 μg/l for GIB sPLA$_2$, 1.8 μg/l (GIIA sPLA$_2$), 6.1 μg/l (GIID sPLA$_2$), 0.3 μg/l (GIIE sPLA$_2$), 3.3 μg/l (GIIF sPLA$_2$), 2.8 μg/l (GV GLA$_2$) and 3.9 μg/l (GX sPLA$_2$), respectively. Since the sample volume was 10 μl, it was possible to detect the sPLA$_2$s in the picogram range (from 3 pg for GIIE sPLA$_2$ to 61 pg for GV sPLA$_2$) by the current TR-FIAs.

To study the day-to-day variations of the TR-FIAs, the same two standard samples containing 12.5 and 50 μg/l of each sPLA$_2$ were analyzed on four different days. The mean interassay cv% ranged from 6.2% in TR-FIA for GIIE sPLA$_2$ to 38.0% in TR-FIA for GIIA sPLA$_2$ (Table 2).

To test the performance of the TR-FIAs in measuring the levels of sPLA$_2$s in tissue extracts, three tissues with different protein concentrations were chosen, namely liver, rectum and thymus. Supernatants of centrifuged extracts of these tissues and serum were spiked with 50 and 100 μg/l of each recombinant sPLA$_2$ protein, and the recovery percentages were

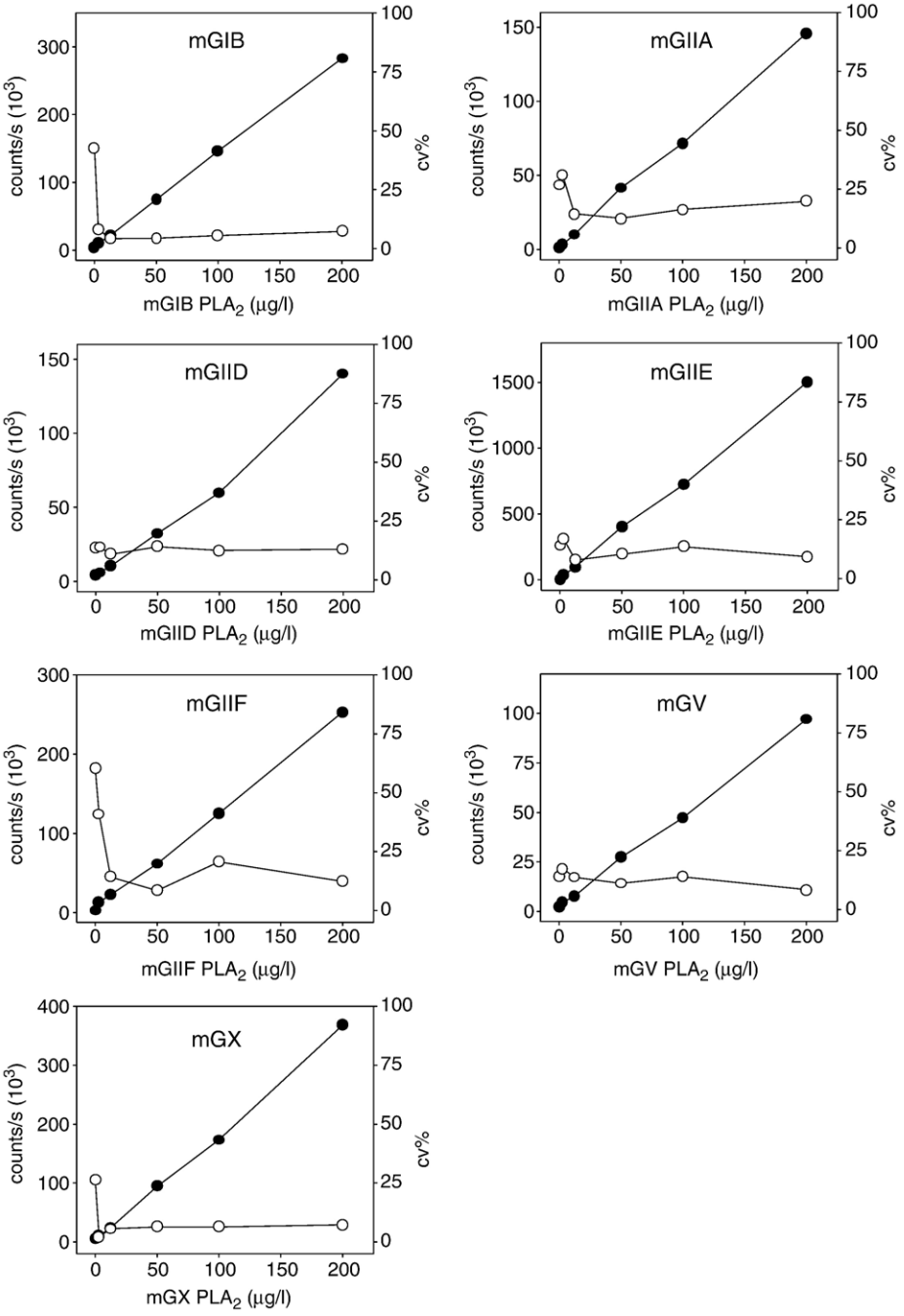

Fig. 1. Standard curves and precision profiles of TR-FIAs for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s. Each data point represents the mean of six measurements. ●=cps vs. µg/l, ○=coefficient of variation (cv%).

determined by TR-FIAs. Mean recoveries ranged from 61% (GIID $sPLA_2$ in the extract of thymus) to 109% (GX $sPLA_2$ in thymus) (Table 3).

3.3. Distribution of $sPLA_2$s in mouse tissues by qPCR

The relative abundance of mRNAs coding for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s was measured by qPCR in several organs/tissues matching those analyzed by TR-FIAs (Fig. 2). To facilitate the comparison of expression between the different $sPLA_2$s in tissues, the data were normalized to the lowest expression levels of $sPLA_2$s that can be accurately measured under our qPCR assays (see Materials and methods). Marked differences were observed between $sPLA_2$s with the highest relative expression levels for GIB and GIIA $sPLA_2$s in the gastrointestinal tract. On average, the lowest relative abundance values were obtained for GV, GX and GIIF $sPLA_2$s.

Table 2
Interassay cv% of the TR-FIAs for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s analyzed on four different days

Conc.	Assay						
	GIB	GIIA	GIID	GIIE	GIIF	GV	GX
12.5 μg/l	16.5	36.4	18.3	12.2	13.8	24.1	15.4
50 μg/l	10.5	38.0	12.5	6.2	16.9	22.9	22.9

Conc. = concentration of the antigen in the sample.

3.4. Distribution of $sPLA_2$s in mouse serum and tissues determined by TR-FIAs

We determined the concentrations of GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s in serum and 24 tissues of three female and three male Balb/c mice (Fig. 3) and three male C57BL/6J mice (Fig. 4). The results are expressed as nanogram of $sPLA_2$ protein per milligram of total protein (ng/mg) in tissue homogenates and as ng/ml in sera. Concentrations of $sPLA_2$s as high as 2100 ng/mg protein (GIIA $sPLA_2$ in small intestine) and 9100 ng/mg protein (GIB $sPLA_2$ in stomach) were measured by current TR-FIAs. The concentrations of $sPLA_2$s below the analytical sensitivities of the assays are not shown. The results of three measurements are given as mean ± S.E.M.

In sera of both Balb/c and C57BL/6J mice, the concentrations of all $sPLA_2$s studied except GX $sPLA_2$ were below the levels of the analytical sensitivities of the current assays. The concentration of GX $sPLA_2$ in sera was only slightly above the value of analytical sensitivity in both mouse lines: 4.4 ng/ml (S.E.M. = 0.95, $n=3$) in C57BL/6J mice and 3.6 ng/ml (S.E.M. = 0.20, $n=6$) in Balb/c mice, respectively ($P=0.61$ between the groups).

To confirm that the natural disruption in the gene coding GIIA $sPLA_2$ reported by Kennedy et al. [74] abolishes the expression of GIIA $sPLA_2$ protein in the tissues of C57BL/6J mice, the levels of GIIA $sPLA_2$ in the tissues of Balb/c (+/+ for endogenous GIIA $sPLA_2$) and C57BL/6J (−/− for GIIA $sPLA_2$) mice were compared. The concentrations of GIIA $sPLA_2$ in Balb/c and C57BL/6J mice were 2090 ng/mg (S.E.M. = 690) vs. 0.16 ng/mg (S.E.M. = 0.033, <analytical sensitivity, AS) in small intestine ($P<0.05$), 15.0 ng/mg (3.6) vs. 0.25 ng/mg (0.11,<AS, $P<0.05$) in colon and 23.8 ng/mg (4.38) vs. 0.35 ng/mg (0.33,<AS, $P<0.05$) in rectum, respectively (Figs. 3 and 4).

Table 3
Analytical recoveries of the TR-FIAs for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s in mouse serum and the extracts of liver, rectum and thymus

Sample	Conc.	Assay						
		GIB	GIIA	GIID	GIIE	GIIF	GV	GX
Serum	50 μg/l	100	78	79	70	89	86	88
	100 μg/l	109	87	107	75	107	106	101
	Mean	**105**	**83**	**93**	**73**	**98**	**96**	**95**
Liver	50 μg/l	78	130	74	73	87	93	89
	100 μg/l	80	115	71	97	75	99	110
	Mean	**79**	**123**	**73**	**85**	**81**	**96**	**99**
Rectum	50 μg/l	74	122	160	75	91	100	64
	100 μg/l	72	90	126	74	96	110	107
	Mean	**73**	**106**	**143**	**74**	**94**	**105**	**86**
Thymus	50 μg/l	78	96	71	102	83	81	103
	100 μg/l	88	77	51	107	82	73	115
	Mean	**83**	**86**	**61**	**104**	**83**	**77**	**109**

Conc. = concentration of the added antigen in the sample.

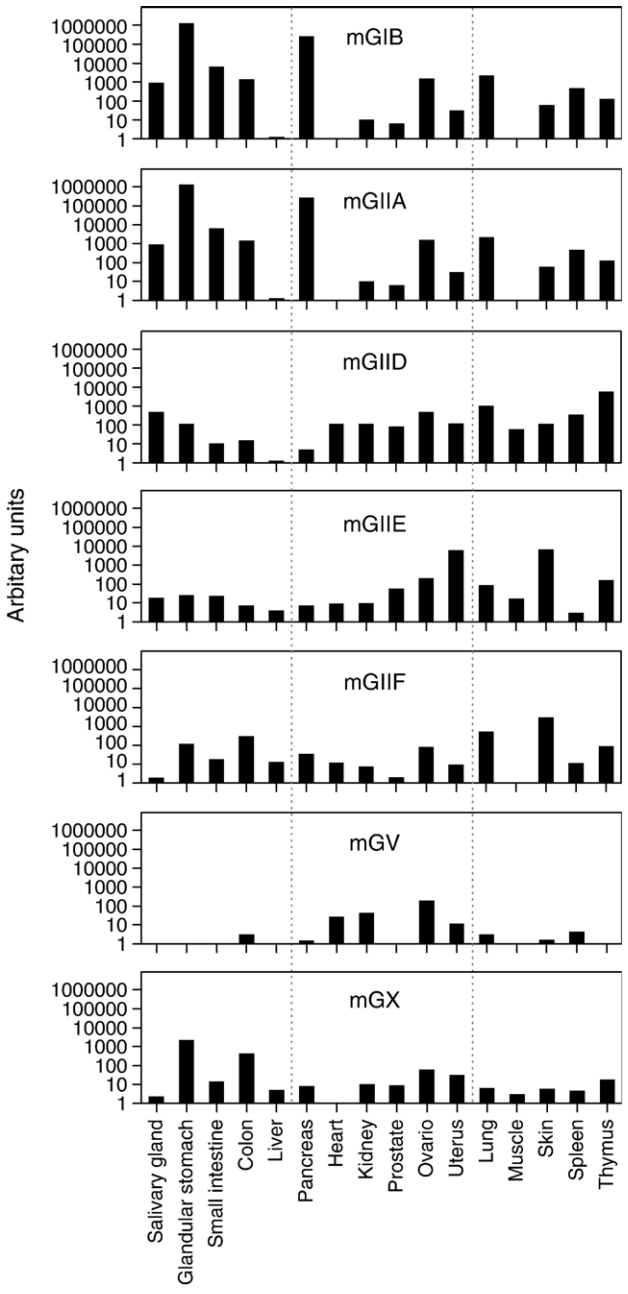

Fig. 2. Distribution of GIB, GIIA, GIID, GIIE, GIIF, GV and GX $sPLA_2$s in mouse tissues by qPCR. Total RNA from the indicated tissues of adult Balb/c mouse were subjected to qPCR using specific sets of $sPLA_2$ primers and the SYBR Green method (see Materials and methods). All the expression data are expressed relative to the expression of 18 S rRNA, which was used as a reference gene. The tissue distribution of $sPLA_2$s was further normalized to the lowest signals measured for $sPLA_2$s in some tissues (Ct values higher than 32). A relative abundance of 1 (arbitrary unit = 1) was assigned to the extremely low and similar expression levels of GIB $sPLA_2$ in the heart and skeletal muscle, GIIA $sPLA_2$ in the spleen, GIID $sPLA_2$ in the liver, GIIF $sPLA_2$ in skeletal muscle, GV $sPLA_2$ in several tissues, and GX $sPLA_2$ in the heart. Note that the data are represented with a log scale.

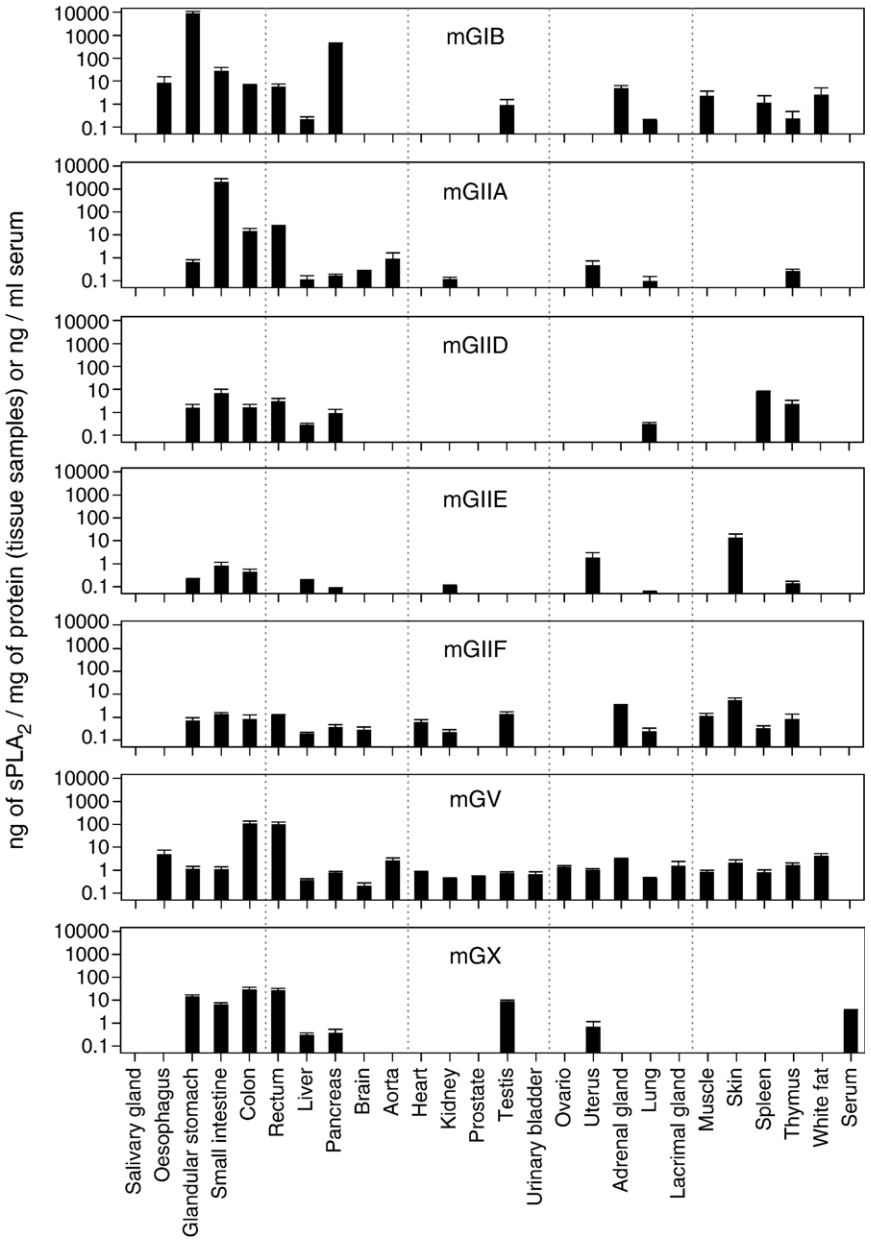

Fig. 3. Distribution of sPLA$_2$s in Balb/c mouse serum and tissues measured by TR-FIAs. The concentrations of sPLA$_2$s in the tissues are expressed as ng of sPLA$_2$/mg protein and in serum as ng of sPLA$_2$/ml serum. Data are represented as mean ± S.E.M. (n = 3 in each group) with a log scale.

To evaluate the possibility that GIIA sPLA$_2$ may be functionally replaced by other sPLA$_2$s in the tissues of C57BL/6J mice, we compared the concentrations of sPLA$_2$s other than GIIA sPLA$_2$ in the tissues of Balb/c and C57BL/6J mice. No statistical differences between the concentrations of sPLA$_2$s other than GIIA sPLA$_2$ in the tissues of Balb/c and C57BL/6J mice were found (data not shown).

4. Discussion

In the current study, we measured the distribution of sPLA$_2$s in mouse tissues at both protein and mRNA levels. For protein expression studies, we developed a novel sensitive TR-FIAs for mouse GIB, GIIA, GIID, GIIE, GIIF, GV and GX sPLA$_2$s based on the use of rabbit polyclonal anti-mouse sPLA$_2$ antibodies. To allow a direct comparison of the expression of sPLA$_2$s at the mRNA versus protein levels, we analyzed the mRNA expression level of the mouse sPLA$_2$s by qPCR. Our study is the first report that determines, with high sensitivity, the protein concentration of a large panel of sPLA$_2$s in serum and tissues of Balb/c and C57BL/6J mice.

The performance of the TR-FIAs developed was evaluated in detail. The specificity of the TR-FIAs was confirmed by testing the cross-reactivity of the antibodies against the non-relevant sPLA$_2$ antigens. The test showed that the antibodies were highly specific. Our results are in agreement with the previous cross-reactivity studies performed by western blot for mouse and human recombinant sPLA$_2$s [32,73]. By using these antibodies, we were

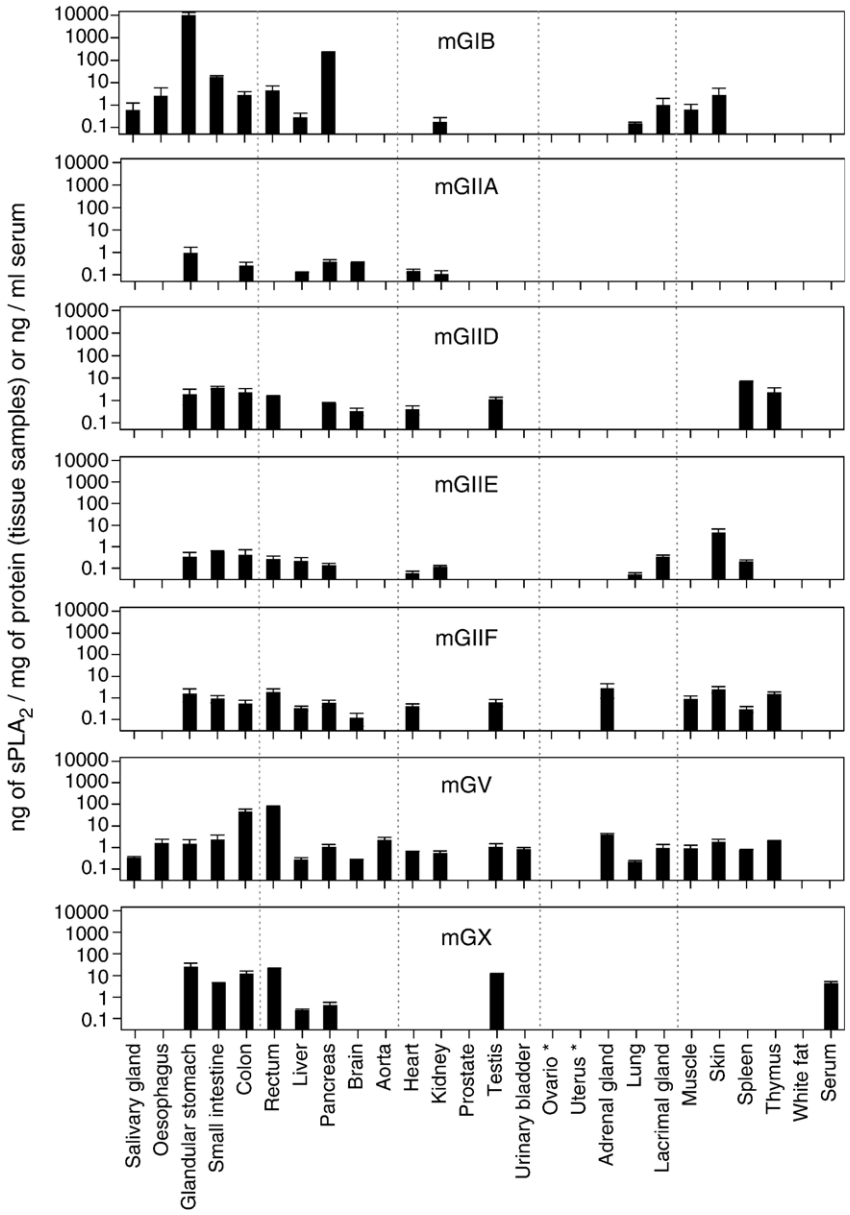

Fig. 4. Distribution of $sPLA_2$s in C57BL/6J mouse serum and tissues measured by TR-FIAs. The concentrations of $sPLA_2$s in the tissues are expressed as ng of $sPLA_2$/mg protein and in serum as ng of $sPLA_2$/ml serum. Data are represented as mean±S.E.M. (n=3 in each group) with a log scale. *=not determined.

able to construct TR-FIA standard curves using recombinant mouse $sPLA_2$ to accurately measure the concentrations of the various subtypes of $sPLA_2$s in mouse serum and tissue homogenates. The standard curves indicated linearity up to 200 μg/l for each $sPLA_2$, allowing the conversion of the cps-values from the fluorometer to $sPLA_2$ concentrations over a wide measurement range. The analytical sensitivities of the current TR-FIAs for mouse $sPLA_2$s varied from 0.3 μg/l for GIIE $sPLA_2$ to 6.1 μg/l for GIID $sPLA_2$. The validity of the TR-FIAs was verified by analytical recovery and reproducibility tests. Analytical recoveries showed that all the current TR-FIAs were suitable for the detection of $sPLA_2$s in serum. Based on the good recoveries also observed in three different tissue homogenates with different protein concentrations, we assumed that the performance of the current TR-FIAs would be reliable in homogenates of other tissues. The low signals around the detection limit of the TR-FIA for GIIA $sPLA_2$ seen in some tissues of both Balb/c and C57BL/6J mice would imply that these signals are not due to GIIA $sPLA_2$ but could be due to other proteins interfering with the assay. Reproducibility was tested by determining the day-to-day variation for each TR-FIA. The variation ranged from 6.2% for TR-FIA for GIIE $sPLA_2$ to 38.0% for TR-FIA for GIIA $sPLA_2$.

While extremely low concentrations of mRNA of $sPLA_2$s can be detected in tissues by qPCR (see below), the detection of $sPLA_2$s in mouse tissues at the protein level by western blotting has been problematic in most cases ([68], Gelb, M.H., Lambeau G. and coworkers, unpublished data). This is presumably due to the relatively low concentrations of these proteins in most human and mouse tissues (exceptions are GIB and GIIA $sPLA_2$s in some tissues). Furthermore, western blots are done under denaturing

conditions that may expose epitopes causing non-specific cross reactivity with non-$sPLA_2$ proteins. Protein denaturation is not a problem when using TR-FIA since sample proteins are in their native state in aqueous solutions. Since the current antibodies were produced against fully folded recombinant $sPLA_2$ [42], TR-FIAs may be more specific than western blotting for the detection of $sPLA_2$s.

Our current results by TR-FIAs and qPCR further support the notion that $sPLA_2$s are expressed in various mouse tissues, as previously proposed in many studies based on mRNA analysis [32,51,52,56,58,63,68]. Indeed, our qPCR results were found to be in fairly good agreement with the above previous data obtained by northern blots or endpoint RT-PCR, and with the *in silico* expression profile suggested by analysis of EST counts for the different mouse $sPLA_2$ genes (http//www.ncbi.nlm.nih.gov/UniGene/). In addition, in most cases, a fairly good correlation was observed between the results obtained by qPCR and TR-FIAs (Figs. 2 and 3). About 60% of our findings in tissues were discovered with both qPCR and TR-FIAs. About one third of the findings were observed with qPCR but not at protein level by TR-FIAs. About 5% of the results were found with TR-FIA but not with qPCR, of which most were results of GV $sPLA_2$ TR-FIA. The discrepancies observed for some $sPLA_2$s between mRNA and protein levels may be due to the extraction method or degradation of the $sPLA_2$ protein depending on the tissue type, or alternatively, to transport of the protein from its site of synthesis to the tissue sampled. Besides, the qPCR method is more sensitive than protein detection methods, even immunoassays, and is capable of revealing extremely low concentrations of mRNA.

Our current results obtained at the mRNA and protein levels clearly indicate that GIB and GIIA $sPLA_2$s are by far the most abundant enzymes, at least in some mouse tissues including stomach and pancreas. This distribution has been also observed in human tissues at the mRNA level [8,17] and in serum and tissues at the protein level [25,47,48,72]. This observation and the fact that GIB and GIIA $sPLA_2$s are the enzymes that have by far the highest specific activities [42] on phospholipid substrate routinely used to detect $sPLA_2$ activity, most certainly explain why these $sPLA_2$s are the only two that have been detected at the protein level in a numerous of earlier studies. Our TR-FIA results indicate that some $sPLA_2$ proteins, like GV and GX $sPLA_2$s, can be expressed at fairly high levels. Their lower specific activities [42] and restricted distribution in mouse or human tissues [12,17,56,77] probably explain why these enzymes have not been detected in the past before their cloning [8,50]. Low protein expression (Figs. 3 and 4) and low or extremely low specific activities of GIID, GIIE and GIIF $sPLA_2$s [42] certainly explain why these $sPLA_2$s have not been detected at the protein level before their cloning [51,52,56].

The best correlation between mRNA and protein expression was observed in the gastrointestinal tract, where almost all findings were observed with both TR-FIAs and qPCR, with few exceptions. Interestingly, the whole gastrointestinal tract contained nearly all the $sPLA_2$s studied, although their concentrations may differ by up to three orders of magnitude. This observation is probably reminiscent of an ancestral digestive function of $sPLA_2$s, as previously suggested for venom $sPLA_2$s [78]. In agreement with a previous study [60], GIB $sPLA_2$ was present at extremely high concentrations in stomach and pancreas whereas GIIA $sPLA_2$ was present at similar concentrations in small intestine. The relatively high concentrations of GIB $sPLA_2$ found in oesophagus, small intestine, colon and rectum could be assumed to originate from the secretion of this enzyme from glandular stomach and pancreas into the gastrointestinal tract. However, our qPCR results suggest that, in fact, GIB $sPLA_2$ is synthesized also in the former organs and thus produced widely in the gastrointestinal tract. GIID, GIIF and GX $sPLA_2$s were distributed throughout the whole gastrointestinal tract, yet at varying levels. GV $sPLA_2$ was present at fairly high levels only in the large intestine. The presence of several $sPLA_2$s in areas of the gastrointestinal tract that match the highest bacterial loads, as well as in the epidermal layer of the skin, further suggest a key role of $sPLA_2$s in innate immunity [82].

Although GIB $sPLA_2$ has long been proposed to act mainly as a digestive enzyme in the gastrointestinal tract [25], results from gene targeting does not support a critical role of this enzyme in phospholipid digestion [26]. This suggests that GIB $sPLA_2$ may be in fact not critical for phospholipid digestion, or that other $sPLA_2$s can compensate for GIB $sPLA_2$ deficiency. As to GIIA $sPLA_2$, an important role has been established for the enzyme in gastrointestinal bacterial defence [36,79,80]. Whether GIID, GV and GX $sPLA_2$s participate in lipid digestion, bacterial defence [81] or lipid mediator release within the gastrointestinal tract warrants future studies using knock-out mice.

Outside the gastrointestinal tract, $sPLA_2$s were detected only occasionally. GIB $sPLA_2$ was found at both mRNA and protein levels in lung, spleen and thymus. The role of GIB $sPLA_2$ in organs other than pancreas and stomach remains unclear, although gene knock-out suggests a role in obesity [26]. GIIA $sPLA_2$ was found in kidney, uterus, lung and thymus at both mRNA and protein levels. Both qPCR and TR-FIA indicated the presence of GIID $sPLA_2$ in spleen and thymus, GIIE $sPLA_2$ in skin and uterus, GIIF $sPLA_2$ in skin, GV $sPLA_2$ in ovary, uterus and skin and GX $sPLA_2$ in uterus. The specific functions of these $sPLA_2$s remain to be established.

Recently, the expression of GIB, GIIA, GIIC, GIID, GIIE, GIIF, GV and GXIIA $sPLA_2$s was detected at the mRNA and protein levels in the different layers of mouse epidermis [32]. By the current TR-FIAs, we confirmed some of these results: we found low concentrations of GIB $sPLA_2$ in extracts of the skin of C57BL/6J mice and GIIE, GIIF and GV $sPLA_2$s in the skin of both Balb/c and C57BL/6J mice. The current TR-FIAs did not detect other $sPLA_2$ proteins in mouse skin. Interestingly, GIIE and GIIF $sPLA_2$s appeared to be expressed at high levels in skin relative to other tissues and to other $sPLA_2$s found in skin. We found mRNAs of all $sPLA_2$s studied in skin, of which the expression of GIIE and GIIF $sPLA_2$s was high also at the mRNA level relative to other $sPLA_2$s. GIIF $sPLA_2$ may participate in lipid mediator release [67], but the native structure and exact biological role of this particular $sPLA_2$ are still unknown.

Recently, $sPLA_2$s were localized by immunohistochemistry in male murine reproductive organs [58]. GIIC, GIID, GIIE, GIIF, GV and GX $sPLA_2$s were found in testis of C57BL/6 mice and GIIA $sPLA_2$ in testis of FVB mice. Our current findings

support most of these observations, i.e., we found GIIF, GV and GX $sPLA_2$s in the testis of both Balb/c and C57BL/6J mice and, in addition, GIID $sPLA_2$ in the testis of C57BL/6J mice. By the current TR-FIA, GIIA $sPLA_2$ was not detected in mouse testis. In mouse prostate, only GV $sPLA_2$ was present at concentrations detectable by the current TR-FIAs, whereas small amounts of mRNAs of all $sPLA_2$s studied, except GV $sPLA_2$, were found in the prostate. In humans, GIIA $sPLA_2$ is present at high concentrations in seminal plasma [83], presumably secreted by prostatic epithelial cells [84].

Kennedy and coworkers [74] suggested that C57BL/6J mice harboring a natural disruption of the GIIA $sPLA_2$ gene can either survive without this $sPLA_2$ or compensate the loss of GIIA $sPLA_2$ activity with other $sPLA_2$s. Since none of the $sPLA_2$s studied here appeared at higher concentrations in the gastrointestinal tract of C57BL/6J mice compared to Balb/c mice, it does not seem that other $sPLA_2$s quantitatively compensate the loss of GIIA $sPLA_2$ in the intestine of C57BL/6J mice under normal conditions. The lack of GIIA $sPLA_2$ protein detectable by the current TR-FIAs in the tissues of C57BL/6J mice is in agreement with the frameshift mutation in the gene coding for GIIA $sPLA_2$ that prevents the production of this enzyme in these mice [74].

Taken together, we describe here novel time-resolved fluoroimmunoassays for mouse $sPLA_2$s. These methods may be particularly useful for accurate measurements of these proteins in mouse tissues and body fluids in various experimental settings linked to inflammation, cancer and other diseases where many $sPLA_2$s have been shown to be up-regulated or down-regulated at the mRNA levels [12,23].

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