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Diversity in secreted PLA_2-IIA activity among inbred mouse strains that are resistant or susceptible to *Apc*$^{Min/+}$ tumorigenesis

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Abstract

The secreted phospholipase A_2 type IIA (*Pla2g2a*) gene was previously identified as a modifier of intestinal adenoma multiplicity in *Apc*$^{Min/+}$ mice. To determine if intestinal secreted phospholipase A_2 ($sPLA_2$) activity was also attenuated in susceptible strains, we developed a sensitive assay to directly quantitate $sPLA_2$ activity in the murine intestinal tract utilizing a fluorescent BODIPY-labeled phospholipid substrate. Here, we report assay conditions that distinguish between secreted and cytosolic PLA_2 enzyme activities in extracts of intestinal tissue. The small intestine exhibited higher activity levels than the large intestine. Consistent with predictions from the $sPLA_2$-IIA gene sequence in inbred strains, we detected low levels of enzyme activity in inbred strains containing $sPLA_2$-IIA mutations; these strains were also associated with greater numbers of intestinal polyps. Additionally, the assay was able to distinguish differences in levels of $sPLA_2$ activity between neoplasia-resistant strains, which were then shown by sequencing to carry variant wild-type $sPLA_2$-IIA alleles. Immunohistochemical analyses of intestinal tissues were consistent with $sPLA_2$-IIA activity levels. This approach enables further studies of the mechanisms of $sPLA_2$ action influencing the development and tumorigenesis of the small intestine and colon in both mice and humans.

Keywords

APC; PLA2; phospholipase; *Mom1*; intestine; cancer; mouse; BODIPY

Introduction

To assay directly enzymatic function and lipid metabolism in live animals, we have developed a family of fluorescent lipid reporters whose spectral characteristics change upon processing by lipid modifying enzymes (Hendrickson *et al.*, 1999; Farber *et al.*, 2001). We

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focused on phospholipase A_2 (PLA_2) activity because of its importance in generating lipid signaling molecules, host defenses, lipid absorption and cancer (MacPhee *et al.*, 1995; Dennis, 1997; Cormier *et al.*, 2000). PLA_2 is a lipolytic enzyme that catalyses the hydrolysis of phospholipids liberating *sn2* fatty acids and lysophospholipids. PLA_2 enzymes have been extensively studied and subdivided into several groups that include, but are not limited to, secreted PLA_2 (Groups IB, IIA, IIC, IID, IIE, IIF, III, V, X and XII—typically low molecular weight, <30 kDa proteins) and cytosolic PLA_2 (Group IVα–IVδ high molecular weight, >80 kDa proteins) (Six and Dennis, 2000; Murakami and Kudo, 2002).

As a first step in developing novel PLA_2 substrates, a number of fluorescent phospholipids were evaluated for their ability to be cleaved by different PLA2s (Farber *et al.*, 1999). Although cytosolic PLA_2 ($cPLA_2\alpha$) exhibits a strong acyl chain preference for polyunsaturated fatty acids, most notably arachidonic acid, the obligate precursor of eicosanoids, BODIPY-labeled phospholipids are excellent substrates for these enzymatic reactions (Farber *et al.*, 1999). Consistent with this observation Dahim *et al.* (2002) found that the BODIPY-labeled phosphatidylcholine (PC) packs similar to PC containing *cis*-unsaturated acyl chains (Dahim *et al.*, 2002). Digestion of PED6 [*N*-((6-(2,4-dinitro-phenyl)amino)hexanoyl)-1-palmitoyl-2-BODIPY-FL-pentanoyl-*sn*-glycerol-3-phosphoethanolamine] in cell culture, zebrafish and mice results in the release of a fluorescent BODIPY acyl chain that reveals organ-specific PLA_2 activity (Farber *et al.*, 2001; Kim *et al.*, 2002a, b).

In this study, we utilized a PC analog that has a BODIPY fluorophore attached to a short (pentanoyl) acyl chain (D3803, Molecular Probes Inc.) to assay PLA_2 activity in intestinal extracts (Farber *et al.*, 1999). High levels of secreted phospholipase A_2 ($sPLA_2$) expression have been previously detected in mammalian small intestine, specifically localized to Paneth cells at the base of the crypts of Lieberkuhn (Mulherkar *et al.*, 1991, 1993). We were particularly interested in this activity because the $sPLA_2$-IIA (*Pla2g2a*) gene is a semidominant modifier locus, *Mom1* (Modifier of *Min1*), of both polyp size and multiplicity along the intestinal tract of $Apc^{Min/+}$ mice (MacPhee *et al.*, 1995; Cormier *et al.*, 1997, 2000). Analysis of inbred strains showed that AKR, MA, CAST, C3H/HeJ, SWR/J, and BALB/c carry *Mom1* resistant alleles ($Mom1^R$), while B6, BTBR and 129/SvJ carry *Mom1* susceptible alleles ($Mom1^S$) (Kennedy *et al.*, 1995; MacPhee *et al.*, 1995; Gould and Dove, 1997; Koratkar *et al.*, 2004). While these studies implicate the protein in the pathway of intestinal neoplasm formation, they fail to demonstrate that $sPLA_2$-IIA enzymatic activity itself is relevant.

In an effort to study intestinal PLA_2 activity in a variety of mouse strains, we established assay conditions that were highly selective for $sPLA_2$-IIA protein. We utilized this assay to not only characterize the levels of activity in wild-type intestine and colon, but to compare these levels to that in mouse strains prone to intestinal polyps. These data demonstrate that $sPLA_2$-IIA activity differences exist between strains, and that activity levels correlate with differences in the amino acid sequence of the $sPLA_2$-IIA protein. Although activity levels are consistent with providing resistance to small intestinal polyps, some strains may not require $sPLA_2$-IIA for protection against polyps in the colon.

Results

Development of an assay for mouse sPLA$_2$ activity using a BODIPY-labeled substrate

We used a fluorescent BODIPY-labeled phospholipid (D3803) as a substrate for phospholipase PLA$_2$ assays. Although this assay was originally developed as a substrate to measure cytosolic phospholipase activity (Farber *et al.*, 1999), it was clear from this earlier work that sPLA$_2$ would also hydrolyse this lipid. Intestinal tissue extracts from AKR/J mice were selected for these assay development studies because it was previously shown that AKR mice carry a wild-type *sPLA$_2$-IIA* gene, in contrast to C57BL/6J (B6) mice which carry a mutant *sPLA$_2$-IIA* gene (Kennedy *et al.*, 1995; MacPhee *et al.*, 1995)

We initially investigated if assay parameters could be identified that would distinguish sPLA$_2$ activity from other PLA$_2$ activities. Towards this end, we found that sPLA$_2$ activity from intestinal tissue extracts exhibited a striking pH optimum (pH 9.0) such that at neutral pH very little activity is detected (Figure 1). The calcium dependence of sPLA$_2$ enzymes has been well established (Balsinde *et al.*, 2002).

Enzymatic properties of secreted and high molecular weight PLA$_2$ enzyme activities

Two competitive inhibitors were used to demonstrate the specificity of our assay for secreted phospholipase activity: (1) methyl arachidonyl fluorophosphate (MAFP), which is a selective, active-site directed, irreversible suicide inhibitor of both Ca^{2+}-dependent and Ca^{2+}-independent high molecular weight phospholipase A$_2$ enzymes (cPLA$_2$-IVα and iPLA$_2$-VI) (Balsinde and Dennis, 1996; Huang *et al.*, 1996; Lio *et al.*, 1996), and (2) *p*-bromophenacyl bromyde (BPB), which is a competitive inhibitor that alkylates the catalytic histidine and causes covalent inactivation of sPLA$_2$ (Gelb *et al.*, 1994). Phospholipase activities from intestinal tissue extracts were determined in the presence of both inhibitors with varying calcium levels and assay buffer pH (6–10).

At high calcium concentrations (~100 and 25 mM EDTA) maximal PLA$_2$ activity was detected at pH 9 (Figure 2, top right) and was inhibited by BPB, but was not inhibited by MAFP. These results confirm that the phospholipase activity detected at pH 9 and 100 mM Ca^{2+} was specifically that of sPLA$_2$, since MAFP is a selective inhibitor of iPLA$_2$-VI and cPLA$_2$-IVα while BPB is a specific inhibitor of sPLA$_2$. However, when the calcium concentration was reduced 10-fold, intestinal PLA$_2$ activity was BPB insensitive (Figure 2 bottom). Under these conditions, addition of MAFP uniformly decreased PLA$_2$ activity at all pH levels compared to controls. Therefore, we can distinguish secreted from high molecular weight PLA2s by altering pH and calcium concentration of the assay buffer containing the BODIPY-labeled phospholipid. Having established the selectivity of this assay, all subsequent sPLA$_2$ determinations were performed at pH 9 with a high calcium concentration (mM).

sPLA$_2$ activity in the small and large intestines in AKR/J mice

sPLA$_2$ activity was then determined along the entire length of the intestinal tract (proximal small intestine to the distal colon) by dissecting samples from four freshly killed animals (Figure 3). sPLA$_2$ activity was significantly higher (three fold, $P<0.01$) in the small intestine

compared to the colon. Additionally, $sPLA_2$ activity appears to increase as one moves from the proximal end to the distal end of the colon (ANOVA, $P<0.01$). Although a similar trend may be present in the small intestine, statistical analyses did not reach significant levels. However, in both the small intestine and colon, there appears to be a drop in activity in the most distal segment.

Demonstration of assay specificity for $sPLA_2$ activity

To prove that the assay conditions could distinguish sPLA2 activity from the activity of other phospholipases, we utilized reciprocal congenic strains (Figure 4). We had previously shown by sequencing and Northern blots that the C57BL/6J strain contains a mutated, null allele of *$sPLA_2$-IIA*−, whereas the AKR strain contains a wildtype allele of *$sPLA_2$-IIA*+ (MacPhee *et al.*, 1995). Our congenic strain B6.AKR *$sPLA_2$-IIA*+/+ has the mutant B6 *$sPLA_2$-IIA*− region replaced by the wildtype AKR *$sPLA_2$-IIA*+ region, with the remainder of the genome being derived from the B6 background. Conversely, our congenic strain AKR.B6 *$sPLA_2$-IIA*−/− mice has the wildtype AKR *$sPLA_2$-IIA*+ region replaced by the mutant B6 *$sPLA_2$-IIA*− region, with the remainder of the genome being derived from the AKR background. The resulting chromosome four regions in representative congenic animals are diagrammed in Figure 4. The $sPLA_2$-IIA activity detected in both AKR and B6.AKR *$sPLA_2$-IIA* +/+ mice is quite robust, with the activity in the congenic B6.AKR *$sPLA_2$-IIA* +/+ mice reaching >80% of the activity levels seen in the control AKR mice. In contrast, the *$sPLA_2$-IIA* activity detected in both B6 and AKR.B6 *$sPLA_2$-IIA*−/− mice was <4% of the activity detected in the control AKR mice.

These results demonstrate that the activity detected on our assay must be due to the congenic *$sPLA_2$* region present on mouse chromosome 4, and not due to other sPLA2s such as *$sPLA_2$-III, $sPLA_2$-X* and *$sPLA_2$-XIIA*, and *$sPLA_2$-XIIB* which are located on mouse chromosomes 11, 16, 3 and 10, respectively (Valentin *et al.*, 1999; Ho *et al.*, 2001; Rouault *et al.*, 2003). The congenic region on mouse chromosome 4 contains five type II *sPLA2s* (*$sPLA_2$-IIA, $sPLA_2$-IIC, $sPLA_2$-IID, $sPLA_2$-IIE*, and *$sPLA_2$-IIF*) along with one type *$sPLA_2$*-V (*Pla2g5*) (www.ensembl.org/Mus_musculus/). It is likely that our assay specifically detects the activity of the $sPLA_2$-IIA protein, because previous studies have demonstrated that $sPLA_2$-IIA is by far the most abundantly expressed $sPLA_2$ in the small intestine of mice (Valentin *et al.*, 1999), whereas the remaining $sPLA_2$ genes on mouse chromosome 4 are expressed in tissues other than the intestines (Valentin and Lambeau, 2000). Furthermore, transgenic mice carrying only a wildtype *$sPLA_2$-IIA* genomic transgene on a pure B6 background exhibit high levels of activity in our assay, consistent with those of wild-type controls (Markova, Silverman, Farber, Siracusa and Buchberg, unpublished observations).

$sPLA_2$ activity determinations in different inbred strains resistant and susceptible to *$Apc^{Min/+}$* intestinal tumorigenesis

Previous studies had demonstrated that several inbred strains carried a wildtype $sPLA_2$-IIA gene while others carried a null $sPLA_2$-IIA gene (Kennedy *et al.*, 1995; MacPhee *et al.*, 1995; Gould and Dove, 1997). Susceptibility to intestinal tumorigenesis in the presence of the $Apc^{Min/+}$ mutation is directly related to the absence of wildtype $sPLA_2$-IIA protein

(MacPhee *et al.*, 1995; Cormier *et al.*, 1997, 2000). A cosmid transgene carrying the sPLA$_2$-IIA+ gene from the AKR/J strain was shown to significantly suppress Apc^{Min}-induced intestinal tumorigenesis on the susceptible B6 background (Cormier *et al.*, 2000). We determined the degree of Apc^{Min}-induced intestinal tumorigenesis in F1 hybrids resulting from matings of different inbred strain females to B6 Apc$^{Min/+}$ males (Table 1). The results show distinct differences in polyp multiplicity between strains (Table 1) (Koratkar *et al.*, 2002). Our data indicate that the B6 and P/J strains were 'susceptible', while the C3H and CAST strains were 'resistant' to polyps. We set out to assay sPLA$_2$ activity in these strains by harvesting tissues from five different places along the intestinal tract: (1) proximal small intestine, (2) middle small intestine, (3) distal small intestine, (4) proximal colon, and (5) the distal colon. As predicted, the susceptible B6 and P/J strains had almost no activity, whereas the resistant strains had measurable, yet different levels of activity, with an apparent hierarchy of AKR>C3H>CAST (Figure 5).

Immunohistochemical localization of the sPLA$_2$-IIA protein in the intestinal tract

Comparison of sPLA$_2$ protein localization and quantitation was performed using immunohistochemistry. An antibody for sPLA$_2$-IIA (see Materials and methods) was applied to sections of both the small intestine and colon of adult B6, P/J, C3H and CAST mice (Figure 6). As expected, samples from the susceptible B6 and P/J strains showed no detectable staining with the sPLA$_2$-IIA antibody in the small intestine and colon, consistent with both strains being homozygous for a null sPLA$_2$-IIA allele (MacPhee *et al.*, 1995). However, the resistant C3H and CAST mice show intense cytoplasmic staining for sPLA$_2$-IIA at the base of small intestinal crypts, which is the site of Paneth cells, consistent with both strains being homozygous for wild-type sPLA$_2$-IIA alleles (MacPhee *et al.*, 1995; Koratkar *et al.*, 2002). Intense cytoplasmic staining was also observed within the Goblet cells of the colon, with staining diminishing as the Goblet cells approached the luminal surface. The staining observed in the C3H strain is consistent with its high levels of sPLA$_2$-IIA activity (Figure 6). CAST mice exhibited dramatically decreased, but positive staining in the Goblet cells of the colon, consistent with the reduced levels of sPLA$_2$ activity detected in the colon of CAST mice (Figure 6). Unexpectedly, the resistant CAST strain showed equivalent staining to C3H in the small intestine, despite its more than 50% lower activity levels (Figure 5). This finding, coupled with the gradient of activity levels (Figure 5), prompted us to examine the amino-acid sequence of the wild-type *sPLA$_2$-IIA* gene in the resistant AKR, C3H, and CAST strains.

Sequencing of the sPLA$_2$-IIA coding region reveals differences in amino acids between mouse strains

Based on the results of activity and immunohistochemistry, the coding region of the *sPLA$_2$-IIA* gene was determined in the AKR, C3H and CAST strains. Figure 7 shows that the CAST strain contains a unique *sPLA$_2$-IIA* allele, resulting from the substitution of at least two nucleotides. This results in the substitution of amino acids within and flanking the critical active site for phospholipid cleavage in the sPLA$_2$-IIA protein (Figure 7). These amino-acid changes may alter the structure and/or substrate binding properties of the active site (Scott *et al.*, 1990), thus resulting in differences in levels of activity, as detected in our assay system (Valentin and Lambeau, 2000).

Discussion

We report the development of assay conditions that distinguish the activity of $sPLA_2$-IIA from the activity of other phospholipases in the intestinal tract of mice. The assay is based upon cleavage of a BODIPY-labeled phospholipid substrate that releases a fluorescent acyl chain after cleavage (Farber *et al.*, 1999). The assay conditions require millimolar levels of calcium, consistent with crystallographic studies that demonstrated that calcium is surrounded by a loop of polypeptide chain in the catalytic domain, where it promotes the binding of phospholipid analogs (Scott *et al.*, 1990). Our data show that maximal intestinal $sPLA_2$ activity is achieved at an alkaline pH of 9 when the Ca^{2+} concentration is 100 mM (Figure 1). Consistent with this observation, purified extracellular phospholipase A2 from human synovial fluid also exhibited highest activity at an alkaline pH in the presence of calcium (Hara *et al.*, 1989). When a micellar preparation of a PC analog is the substrate for porcine pancreatic $sPLA_2$, higher Ca^{2+} is required at alkaline pH, but not at neutral pH (Gelb *et al.*, 1995). It is hypothesized that one effect of enzyme-bound Ca^{2+} is to increase the p*K*a to 9.3 only when Ca^{2+} is present, presumably because of a shift in the p*K*a of the N-terminal alanine (Gelb *et al.*, 1995). Regardless of the exact mechanism, these assay conditions are ideally suited to assay $sPLA_2$-IIA activity from complex biological tissue extracts.

The AKR strain was chosen for detailed analysis primarily because it was shown to carry a wild-type *$sPLA_2$-IIA* gene, which confers resistance against *Apc^{Min}*-induced intestinal polyps (MacPhee *et al.*, 1995; Gould *et al.*, 1996; Cormier *et al.*, 1997, 2000). Overall activity in the AKR small intestine was significantly three-fold higher ($P<0.01$) than in the colon (Figure 3). Furthermore, a trend of increasing activity from proximal to distal small intestine and from proximal to distal colon was evident (with the final section of each organ showing a dramatic decrease in activity). This trend is consistent with, and most likely reflects, the increased density of villi along the proximal–distal axis of the small intestine (as well as the colon). In addition, it explains the detection of $sPLA_2$ as a modifier gene, since the largest number of polyps arise in the distal portion of the mouse small intestine in susceptible strains, where the highest levels of $sPLA_2$ activity are detected in resistant strains.

To examine more fully the levels of $sPLA_2$-IIA activity between different mice, we compared susceptible strains (B6 and P/J) with resistant strains (AKR, C3H and CAST) (Figures 4 and 5 and Table 1). The absence of $sPLA_2$ activity and negative immunohistochemical staining along the intestinal tract in the B6 and P/J strains was expected, since both strains carry null *$sPLA_2$-IIA* genes (MacPhee *et al.*, 1995). In contrast, the enzymatic assay was able to distinguish activity levels between the resistant AKR, C3H and CAST strains (Koratkar *et al.*, 2002; Koratkar *et al.*, 2004). However, neither C3H nor CAST mice exhibited the $sPLA_2$ activity levels seen in the middle small intestine of AKR mice (used as a control). Furthermore, the CAST strain exhibited significantly lower levels of $sPLA_2$ activity than C3H along the entire intestinal tract. These findings demonstrate the sensitivity of the assay in quantitating activity differences among inbred strains carrying wild-type *$sPLA_2$-IIA* alleles. However, overall amount of $sPLA_2$-IIA protein (as evidenced by immunohistochemical assays) did not appear to vary between the AKR, C3H and CAST strains in the small intestine (Figure 6). This observation led us to sequence the coding

region of sPLA$_2$-IIA; the results show that the CAST strain has amino-acid differences compared to AKR and C3H (Figure 7). This difference could result in the hierarchy of activity noted between the strains (Figure 5), whereas overall protein expression levels would remain equivalent. A likely hypothesis is that these allelic differences influence the structure of the active site for phospholipid cleavage.

The low level of sPLA$_2$-IIA activity and protein detected in the colon of CAST mice is intriguing. Of the initial crosses that established the presence of the *Mom1* locus in inbred strains carrying the *Apc^{Min}* mutation, CAST was the strain with the lowest LOD score (Dietrich *et al.*, 1993). Furthermore, we have shown that the CAST strain does not need a *Mom1* locus to provide significant resistance to polyp formation in the colon, although absence of the *sPLA$_2$-IIA* region results in a 2–3- fold increase in polyp number in the small intestine of CASTB6F1 *Apc^{Min}* hybrids (Koratkar *et al.*, 2004). In addition, wild-type sPLA$_2$-IIA protein can significantly decrease polyp number in the colon of B6 mice (Cormier *et al.*, 1997; Cormier *et al.*, 2000). However, absence of the *sPLA$_2$-IIA* gene has little effect on colon polyp number in C3HB6F1 *Apc^{Min}* hybrids, indicating that the C3H genome has other genes that impact colon tumorigenesis (Koratkar *et al.*, 2004). It is also interesting that the P/J strain, which carries a null *sPLA$_2$-IIA* allele, exhibited only a 9% incidence of polyps in the colon (Table 1). Therefore, the CAST, C3H, and P/J strains must have modifier genes, other than *sPLA$_2$-IIA*, that result in significantly lower polyp numbers in the colon. This sensitive enzymatic assay can be used to examine different levels of sPLA$_2$ activity and their relationship to polyp formation on the same genetic background, thereby eliminating the complicating factor of modifier genes. It will also be interesting to apply this assay to a number of different human tissues and disease states; the assay may be useful for measuring activity levels in human patients to determine if there exists a relationship between activity levels and prognosis or response to therapy.

Materials and methods

Mice

AKR/J (AKR), CAST/Ei (CAST), C3H/HeJ (C3H), C57BL/6J (B6) and P/J mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the Thomas Jefferson University (TJU) AAALAC-accredited Facility. Mice were fed laboratory autoclavable rodent diet 5010 (PMI Nutrition International, Inc., Brentwood, MO, USA) for at least 2 weeks before euthanasia. All cages, food, bedding and water were autoclaved prior to use.

Tissue extraction

Mice were euthanized by CO_2 asphyxiation. The entire intestinal tract was immediately dissected. Individual pieces of the small and large intestines were taken and gently scraped to remove contents. Each piece was placed directly into liquid nitrogen; frozen tissue was weighed (~50 μg) and extraction buffer (100 μl buffer/10 μg tissue: 50 mM tris-HCl containing 25 mM EDTA, pH 9.0) was added, then the tissue was briefly sonicated. Aliquots (5 μl) of crude extract solution were used immediately and the remainder stored at −80 °C.

Protein quantity was determined using the BSA Protein Assay Kit (Pierce, Rockford, IL, USA).

Assay for determination of PLA_2 activity

Assays were performed using the fluorescent PC substrate-2-(4, 4-difluoro-5, 7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine) (D3803, Molecular Probes Inc., Eugene, OR, USA) similar to a method previously described (Farber *et al.*, 1999). D3803 was purified by thin layer chromatography (TLC) to remove low abundance BODIPY-labeled degradation products: 1 mg of D3803 was dissolved in 200 μl of methanol and chloroform (1 : 1). Aliquots were spotted on silica gel plates (Whatman) for TLC and developed (chloroform : methanol : H_2O—35 : 7 : 0.7). The purified substrate was scraped from the plate and eluted with 3–5 methanol washes. Aliquots were stored in methanol at −20 °C. The concentration of D3803 was determined at 504 nm (E = 91 800/cm/M).

Assay buffer was prepared by adding purified substrate to extraction buffer (10 – 15 μg substrate/85 μl reaction) containing either 10 or 100 mM $CaCl_2$. Reactions were started by adding assay buffer (90 μl), to 20 × -diluted tissue extract (5 μl, ~1 – 3 μg of protein), and incubated at 37°C for 15 min. To stop the reaction, 270 μl of methanol : chloroform (1 : 2) was added. After vigorous mixing and a short centrifugation, the organic layer (50 μl) was spotted on a TLC plate. Plates were developed in toluene : ether : ethanol : H_2O (25 : 20: 1 : 0.1) and quantified using a laser scanner (Storm, Amersham Biosciences). Relative fluorescence intensity was determined by analysis of the scanned image (ImageQuant, Amersham Biosciences). The relative activity was calculated as a percentage of substrate cleaved/μg of total protein.

Free Ca^{2+} concentration

MaxChelator (v. 2.4) was used to calculate the free Ca^{2+} concentration (www.stanford.edu/~capatton/downloads.htm). This program incorporates the pH, temperature, and ionic strength of the buffer.

sPLA$_2$-IIA immunohistochemistry

Mice were euthanized by CO_2 asphyxiation. Samples of intestinal tissues were immediately dissected, cleared of residual debris, and placed in 10% buffered formalin. Samples were embedded in paraffin within 24 h. Sections (5 μm) were cut for hematoxylin and eosin staining as well as for immunohistochemistry. An antibody to the full length $sPLA_2$-IIA protein (Mulherkar *et al.*, 1991) was used (1 : 5000 dilution) for standard peroxidase-based immunohistochemistry. Briefly, intestinal tissue sections were deparaffinized and rehydrated through graded ethanol series. Endogenous peroxidases were inactivated by treating the slides with methanol peroxidase. Immunohistochemistry was performed with Vectastain ABC kit used in accordance with manufacturers instructions (Vector Laboratories, Burlingame, CA, USA).

Generation of reciprocal congenic lines using AKR/J and C57BL/6J mice

A standard breeding and selection protocol was followed to derive the congenic lines (Silver, 1995). The B6.AKR *sPLA$_2$-IIA*$^+$ congenic line was obtained from an intercross of B6 and AKR mice. Hybrid F1 offspring were backcrossed to B6 mice. N2 and subsequent N# offspring were genotyped to identify pups carrying the selected AKR *sPLA$_2$-IIA*$^+$ allele along with flanking loci (*D4Mit338–D4Mit127*) (http://www.broad.mit.edu/resources.html), leaving the remainder of the genome homozygous for B6 alleles. Conversely, the AKR.B6 *sPLA$_2$-IIA*$^-$ congenic line was obtained from an intercross of B6 and AKR mice. Hybrid F1 offspring were backcrossed to AKR mice. N2 and subsequent N# offspring were genotyped to identify pups carrying the selected B6 *sPLA$_2$-IIA*$^-$ allele along with flanking loci, leaving the remainder of the genome homozygous for AKR alleles. To ensure the integrity of the *sPLA$_2$-IIA* region during transfer onto the recipient background, we analysed markers at and flanking the *sPLA$_2$-IIA* gene. Intercrosses of heterozygous backcross mice led to the birth of the homozygous congenic animals used in these studies.

Genotyping for the *sPLA$_2$-IIA* gene and flanking loci on mouse chromosome 4

Genomic DNAs were isolated from tail biopsies for PCR analysis (Koratkar *et al.*, 2002, 2004) using SSLP primer pairs purchased from Invitrogen (Huntsville, AL) and/or primer pairs synthesized by the KCC Nucleic Acids Facility. Markers were analysed using standard PCR conditions, electrophoresed on 3% agarose gels in 1XTBE, and visualized by EtBr staining.

Sequencing of *sPLA$_2$-IIA* transcripts

Total RNA was prepared as described (MacPhee *et al.*, 1995). Amplification of *sPLA$_2$-IIA* mRNA was performed using the Titan One Tube RT-PCR kit (Roche Applied Science, Indianapolis, IN). The primer set for amplification was used at an annealing temperature of 54°C for 35 cycles. The 5′ primer (GAC AGC ATG AAG GTC CTC CTG) anneals shortly past the translation start site, whereas the 3′ primer (TGA TGG GAG ACG CGC ATG TC) anneals in the 3′ untranslated region. The reaction yields a 482 bp product that was sequenced using the same primers as in the RT-PCR, with a 377 DNA Sequencer from ABI Prism (Foster City, CA) by the KCC Nucleic Acids Facility.

To sequence the 5′ end of the *sPLA$_2$-IIA* gene, PCR amplification of 200 ng genomic DNA was performed. The 5′ primer (GCC TCT GGG GCT GCT AAG TCA GAC) and the 3′ primer (GGC AGG CAT CTC AGG GTC TTA CC) amplified an 840 bp product that included exons 1–3 of the *sPLA$_2$-IIA* gene. The PCR protocol consisted of an initial 94°C step for 4 min, followed by 30 amplification cycles at 94°C for 30 s, 56°C for 60 s, and 72°C for 60 s, with a final extension step at 72°C for 7 min. The amplified product was sequenced by the KCC Nucleic Acids Facility using the same primers as for the initial amplification.

Assessment of polyp number along the intestinal tract

Apc$^{Min/+}$ F1 progeny generated from crosses between B6, P/J, C3H and CAST females mated to B6 *Apc$^{Min/+}$* males were aged and then euthanized by CO_2 asphyxiation. The small intestine and colon were dissected and cleared of residual debris. Polyps were counted using a Nikon SMZ-U dissection scope (15 × magnification).

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References

1. Balsinde J, Dennis EA. J. Biol. Chem. 1996; 271:31937–31941. [PubMed: 8943239]
2. Balsinde J, Winstead MV, Dennis EA. FEBS Lett. 2002; 531:2–6. [PubMed: 12401193]
3. Cormier RT, Bilger A, Lillich AJ, Halberg RB, Hong KH, Gould KA, Borenstein N, Lander ES, Dove WF. Oncogene. 2000; 19:3182–3192. [PubMed: 10918573]
4. Cormier RT, Hong KH, Halberg RB, Hawkins TL, Richardson P, Mulherkar R, Dove WF, Lander ES. Nat. Genet. 1997; 17:88–91. [PubMed: 9288104]
5. Dahim M, Mizuno NK, Li X-M, Momsen WE, Momsen MM, Brockman HL. Biophys. J. 2002; 83:1511–1524. [PubMed: 12202376]
6. Dennis EA. Trends Biochem. Sci. 1997; 22:1–2. [PubMed: 9020581]
7. Dietrich WF, Lander ES, Smith JS, Moser AR, Gould KA, Luongo C, Borenstein N, Dove W. Cell. 1993; 75:631–639. [PubMed: 8242739]
8. Farber SA, Olson ES, Clark JD, Halpern ME. J. Biol. Chem. 1999; 274:19338–19346. [PubMed: 10383445]
9. Farber SA, Pack M, Ho SY, Johnson ID, Wagner DS, Dosch R, Mullins MC, Hendrickson HS, Hendrickson EK, Halpern ME. Science. 2001; 292:1385–1388. [PubMed: 11359013]
10. Gelb MH, Jain MK, Berg OG. FASEB J. 1994; 8:916–924. [PubMed: 8088457]
11. Gelb MH, Jain MK, Hanel AM, Berg OG. Annu. Rev. Biochem. 1995; 64:653–688. [PubMed: 7574497]
12. Gould KA, Dietrich WF, Borenstein N, Lander ES, Dove WF. Genetics. 1996; 144:1769–1776. [PubMed: 8978062]
13. Gould KA, Dove WF. Proc. Natl. Acad. Sci. USA. 1997; 94:5848–5853. [PubMed: 9159163]
14. Hara S, Kudo I, Chang HW, Matsuta K, Miyamoto T, Inoue K. J. Biochem. (Tokyo). 1989; 105:395–399. [PubMed: 2732214]
15. Hendrickson HS, Hendrickson EK, Johnson ID, Farber SA. Anal. Biochem. 1999; 276:27–35. [PubMed: 10585741]
16. Ho IC, Arm JP, Bingham CO 3rd, Choi A, Austen KF, Glimcher LH. J. Biol. Chem. 2001; 276:18321–18326. [PubMed: 11278438]
17. Huang Z, Payette P, Abdullah K, Cromlish WA, Kennedy BP. Biochemistry. 1996; 35:3712–3721. [PubMed: 8619991]
18. Kennedy BP, Payette P, Mudgett J, Vadas P, Pruzanski W, Kwan M, Tang C, Rancourt DE, Cromlish WA. J. Biol. Chem. 1995; 270:22378–22385. [PubMed: 7673223]
19. Kim YJ, Kim KP, Han SK, Munoz NM, Zhu X, Sano H, Leff AR, Cho W. J. Biol. Chem. 2002a; 277:36479–36488. [PubMed: 12124392]
20. Kim YJ, Kim KP, Rhee HJ, Das S, Rafter JD, Oh YS, Cho W. J. Biol. Chem. 2002b; 277:9358–9365. [PubMed: 11777916]
21. Kirtane BM, Mulherkar R. J. Biosci. 2002; 27:489–494. [PubMed: 12381872]
22. Koduri RS, Gronroos JO, Laine VJ, Le Calvez C, Lambeau G, Nevalainen TJ, Gelb MH. J. Biol. Chem. 2002; 277:5849–5857. [PubMed: 11694541]
23. Koratkar R, Pequignot E, Hauck WW, Siracusa LD. Cancer Res. 2002; 62:5413–5417. [PubMed: 12359746]
24. Koratkar R, Silverman KA, Pequignot E, Hauck WW, Buchberg AM, Siracusa LD. Genomics. 2004; 84:844–852. [PubMed: 15475263]
25. Lio YC, Reynolds LJ, Balsinde J, Dennis EA. Biochim. Biophys. Acta. 1996; 1302:55–60. [PubMed: 8695655]

26. MacPhee M, Chepenik KP, Liddell RA, Nelson KK, Siracusa LD, Buchberg AM. Cell. 1995; 81:957–966. [PubMed: 7781071]
27. Mulherkar R, Desai SJ, Rao RS, Wagle AS, Deo MG. Histochemistry. 1991; 96:367–370. [PubMed: 1787109]
28. Mulherkar R, Rao R, Rao L, Patki V, Chauhan VS, Deo MG. FEBS Lett. 1993; 317:263–266. [PubMed: 8425615]
29. Murakami M, Kudo I. J. Biochem. (Tokyo). 2002; 131:285–292. [PubMed: 11872155]
30. Murakami M, Nakatani Y, Kudo I. J. Biol. Chem. 1996; 271:30041–30051. [PubMed: 8939951]
31. Rouault M, Bollinger JG, Lazdunski M, Gelb MH, Lambeau G. Biochemistry. 2003; 42:11494–11503. [PubMed: 14516201]
32. Scott DL, Otwinowski Z, Gelb MH, Sigler PB. Science. 1990; 250:1563–1566. [PubMed: 2274788]
33. Silver, LM. Mouse Genetics; Concepts and Applications. Oxford University Press; New York: 1995.
34. Six DA, Dennis EA. Biochim. Biophys. Acta. 2000; 1488:1–19. [PubMed: 11080672]
35. Valentin E, Ghomashchi F, Gelb MH, Lazdunski M, Lambeau G. J. Biol. Chem. 1999; 274:31195–31202. [PubMed: 10531313]
36. Valentin E, Lambeau G. Biochim. Biophys. Acta. 2000; 1488:59–70. [PubMed: 11080677]

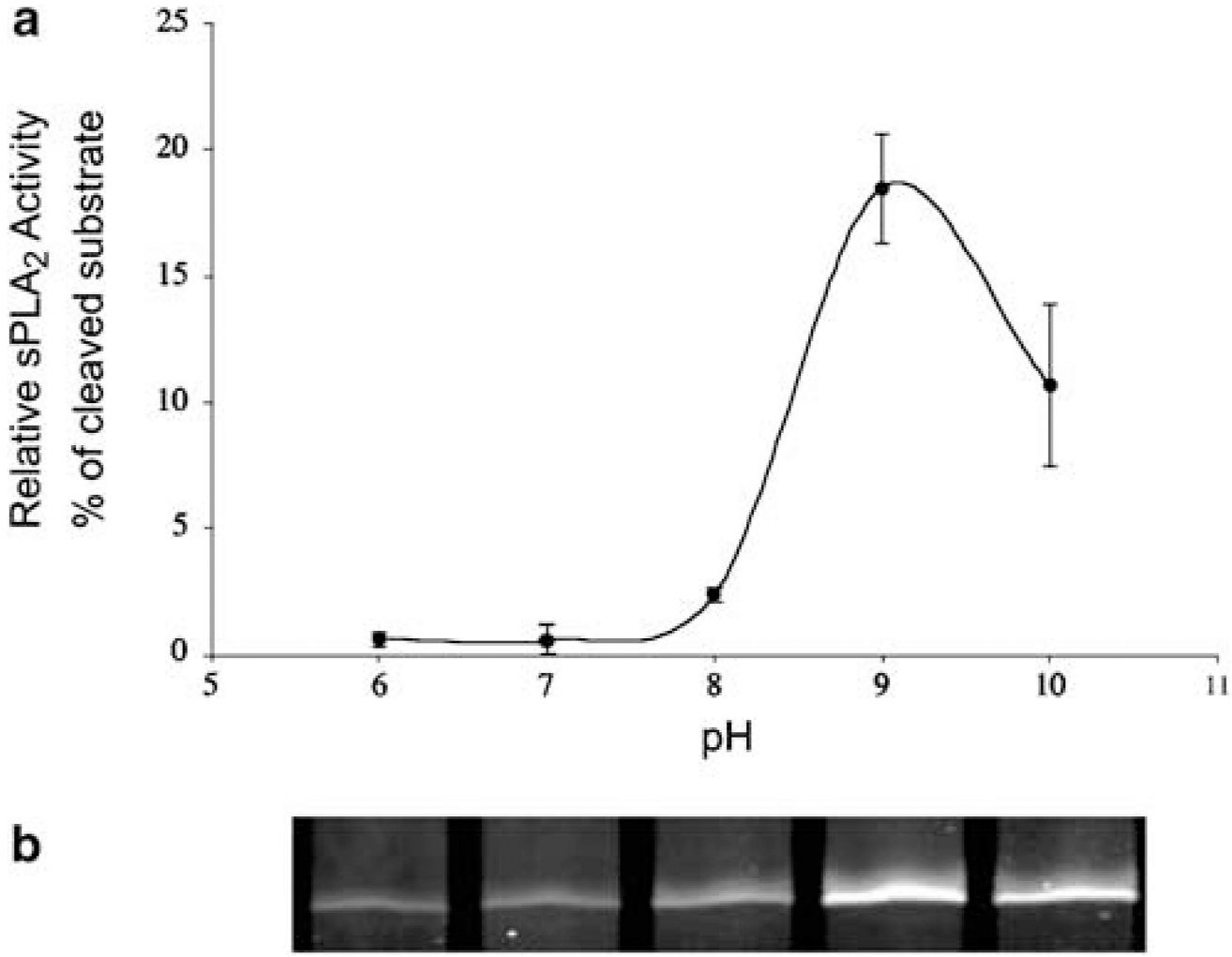

Figure 1.

Effect of pH on secreted PLA_2 activity. Samples were taken from the same region of the distal small intestine of three adult AKR/J male mice. (**a**) Relative PLA_2 activity was expressed as a percentage of cleaved substrate (D3803). Data represent the mean±s.d. at each pH is shown. The time of incubation was 15 min. (**b**) A representative original thin layer chromatography plate with fluorescent products from the same sample analysed at different pH levels. The graph shows that a pH of 9 provides the greatest activity

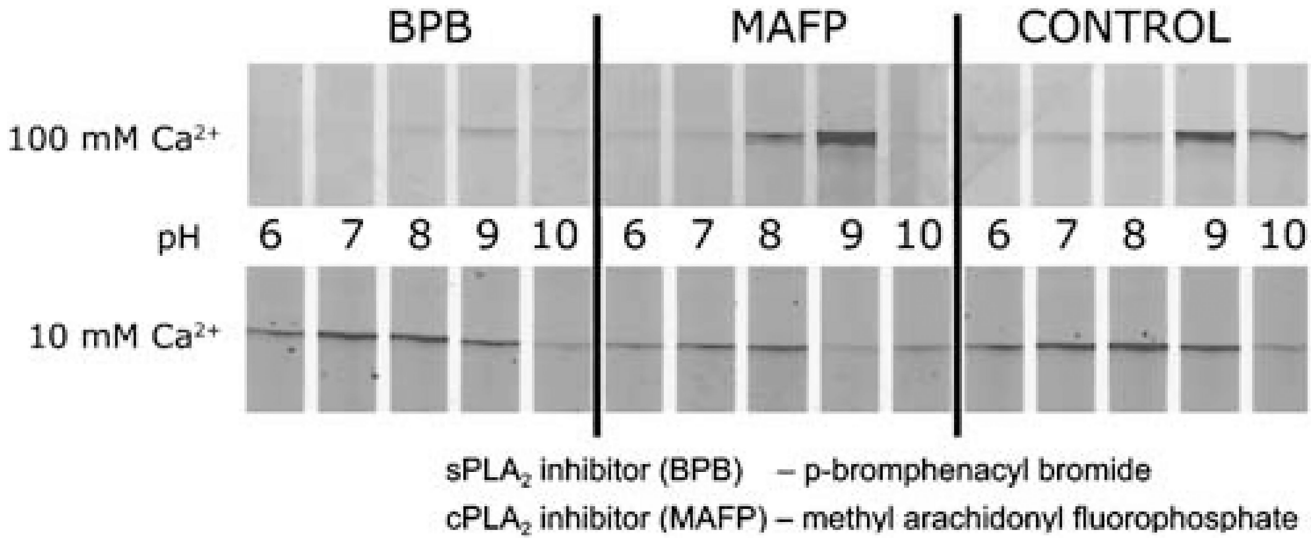

Figure 2.

Calcium concentration and pH can distinguish between secreted and cytosolic PLA_2 enzyme activities. Representative original thin layer chromatography plates with fluorescent products from the same sample analysed at different pH levels and calcium concentrations. Samples were taken from the same region of the distal small intestine of an adult AKR/J male mouse. The reactions for the first plate were performed at 100 mM Ca^{2+} and reactions for the second plate were performed at 10 mm Ca^{2+}. An inhibitor of secreted phospholipase $sPLA_2$ activity (*p*-bromphenacyl bromide, BPB) and an inhibitor of $cPLA_2\alpha$ activity (methyl arachidonyl fluorophosphate, MAFP) were used. The level of the activity at pH 6 – 10 was determined in the absence (control) and presence of these specific inhibitors. Maximum activity was achieved at the higher concentration of Ca^{2+} and a pH of 9

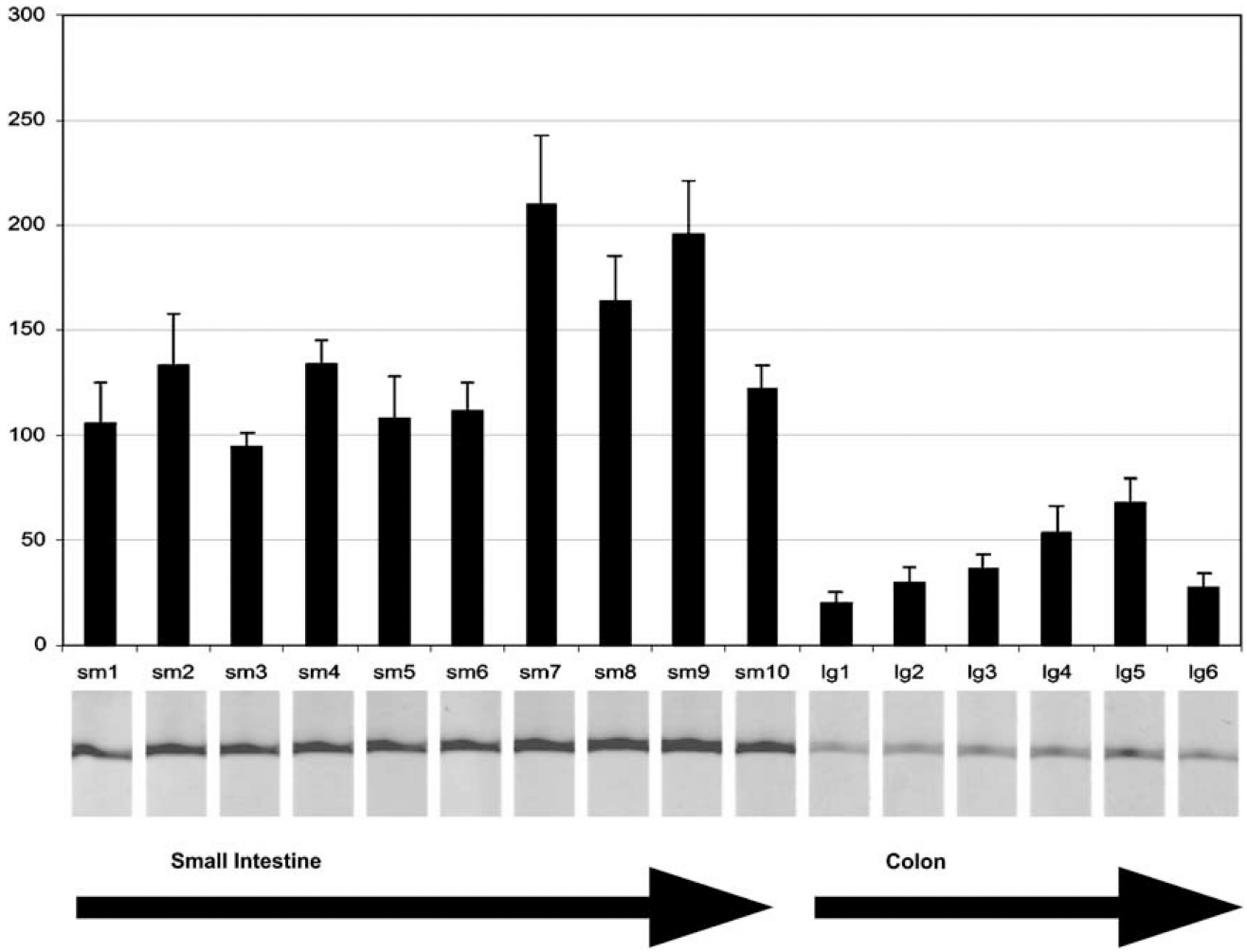

Figure 3.

Secretory PLA_2 activity shows dramatic differences between the small and large intestines in AKR/J mice. Pieces of the small and large intestines were taken at equidistant intervals from each mouse for PLA_2 activity determinations (each assay contains equivalent protein levels). Four 6 – 8-week-old AKR/J males were used in these experiments. (**a**) Pieces from the small intestine are represented by sm1 – sm10, with sm1 representing the most proximal section. Pieces from the large intestine are represented by lg1 – lg6, with lg1 representing the most proximal section. D3803 was repurified before use and dissolved in assay buffer (see Materials and methods). To normalize the four experiments, the average activity for a representative piece was found by totaling the activity from all pieces and dividing by 16. Relative activity for each piece was then expressed as a percentage of the average. Data represent mean±s.d., $n = 3 – 4$. (**b**) A representative original thin layer chromatography plate with fluorescent products from each section of the small and large intestines. The last sample represents a negative control that is the reaction blank, which contains all components of the reaction, but no intestinal extract

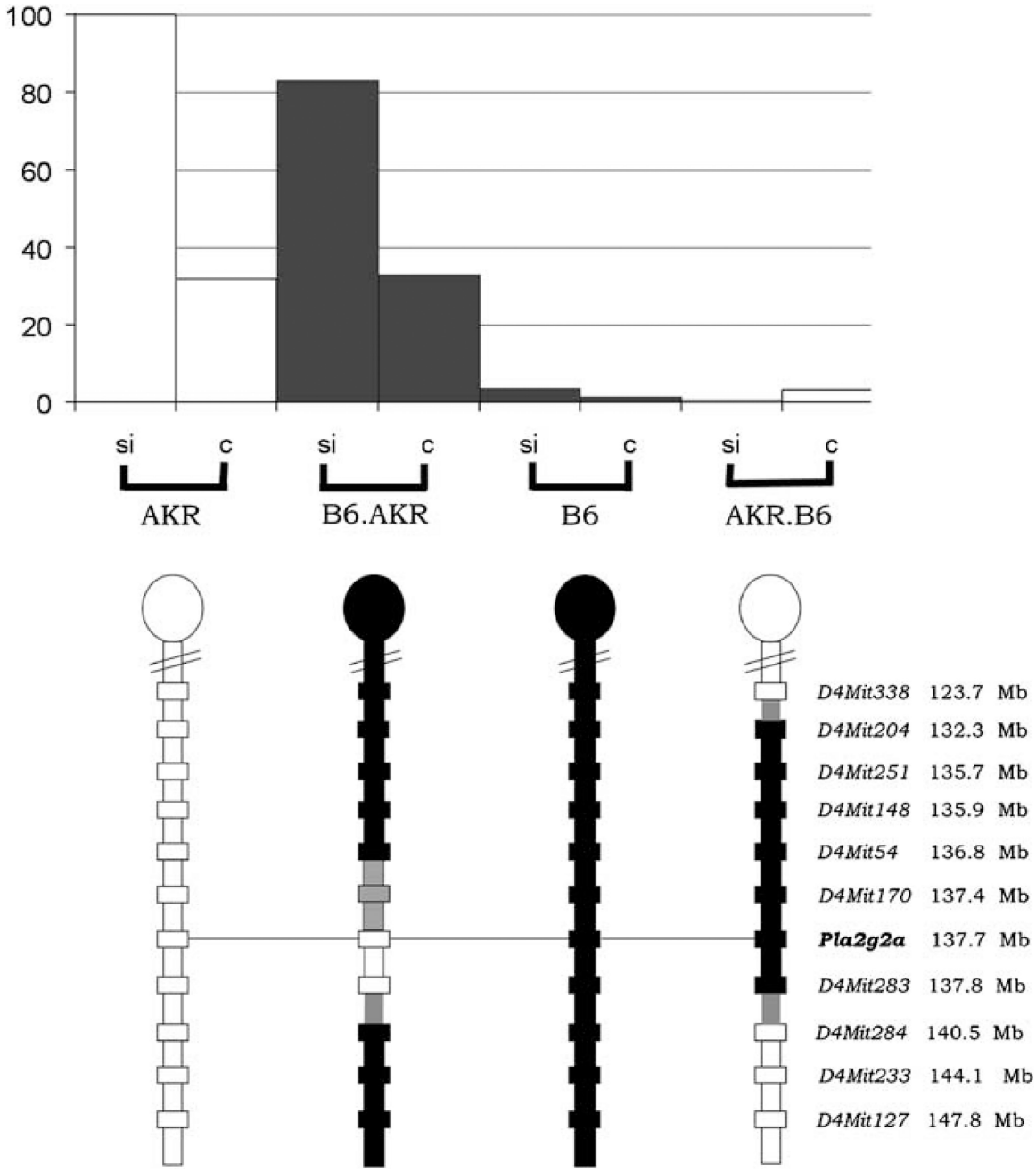

Figure 4.

Comparison of $sPLA_2$ activity levels in the intestinal tract of reciprocal congenic strains. Adult male mice at ~3 months of age were assayed for $sPLA_2$ activity levels. Tissue samples were taken from the middle of the small intestine (si) and the middle of the distal colon (c). Each sample was assayed in duplicate and the graphs represent the average values. The % activity is shown on the *y*-axis; the activity level of the si in the control AKR mouse was set at 100%. The control and congenic chromosome 4 present in each mouse is shown below the graph of their respective activity assays. The B6.AKR *$sPLA_2$-IIA* +/+ and AKR.B6 *$sPLA_2$-IIA* −/− congenic mice were at the N15F4 and N13F3 and backcross generations. Molecular

markers used to genotype the mice are shown to the right of the chromosomes, along with their Megabase positions in version 33 from http://www.ensembl.org. Black represents B6 alleles, white represents AKR alleles, and gray represents heterozygous regions and/or regions of recombination between the donor congenic segment and the recipient chromosome 4. The presence of high levels of activity in the control AKR *sPLA$_2$-IIA* +/+ and congenic B6.AKR *sPLA$_2$-IIA* +/+ mice is consistent with the fact that both mice carry wild-type *sPLA$_2$-IIA* alleles (Kennedy *et al.*, 1995; MacPhee *et al.*, 1995). The minimal activity in the control B6 *sPLA$_2$-IIA*−/− and congenic AKR.B6 *sPLA$_2$-IIA* −/− mice is consistent with the fact that both mice carry null *sPLA$_2$-IIA* alleles (Kennedy *et al.*, 1995; MacPhee *et al.*, 1995)

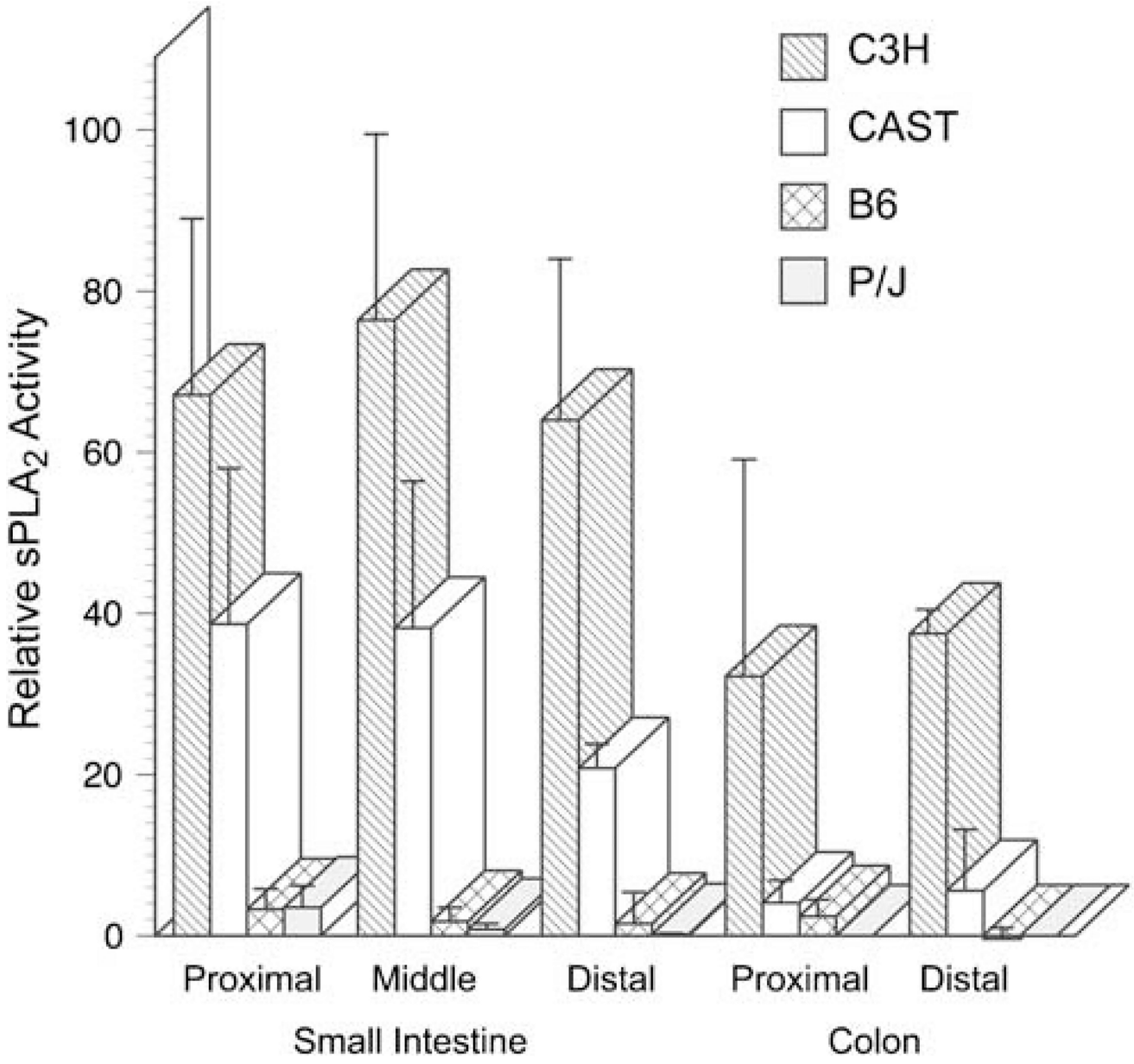

Figure 5.

Comparison of secreted PLA_2 activity between the susceptible C57BL/6J and P/J strains versus the resistant C3H/HeJ and CAST/Ei strains. Tissue specimens from the proximal (pi), middle (mi) and distal (di) small intestine along with the proximal (pc) and distal (dc) large intestine were taken from three 6–8-week-old mice from each of four inbred strains. A single sample from the middle small intestine of an adult AKR/J male mouse was used as a positive control, whereas a single sample from the middle small intestine of a C57BL/6J male mouse was used as a negative control. For these experiments, 50 μg of substrate were used per reaction. To compare levels of activity between resistant and susceptible strains, the activity level for the AKR/J control was normalized to 100%. The activity of each sample is expressed as the percentage of $sPLA_2$ activity compared to the AKR/J control. The inbred strain symbols are: C3H/HeJ (C3H), CAST/Ei (CAST), C57BL/6J (B6) and P/J

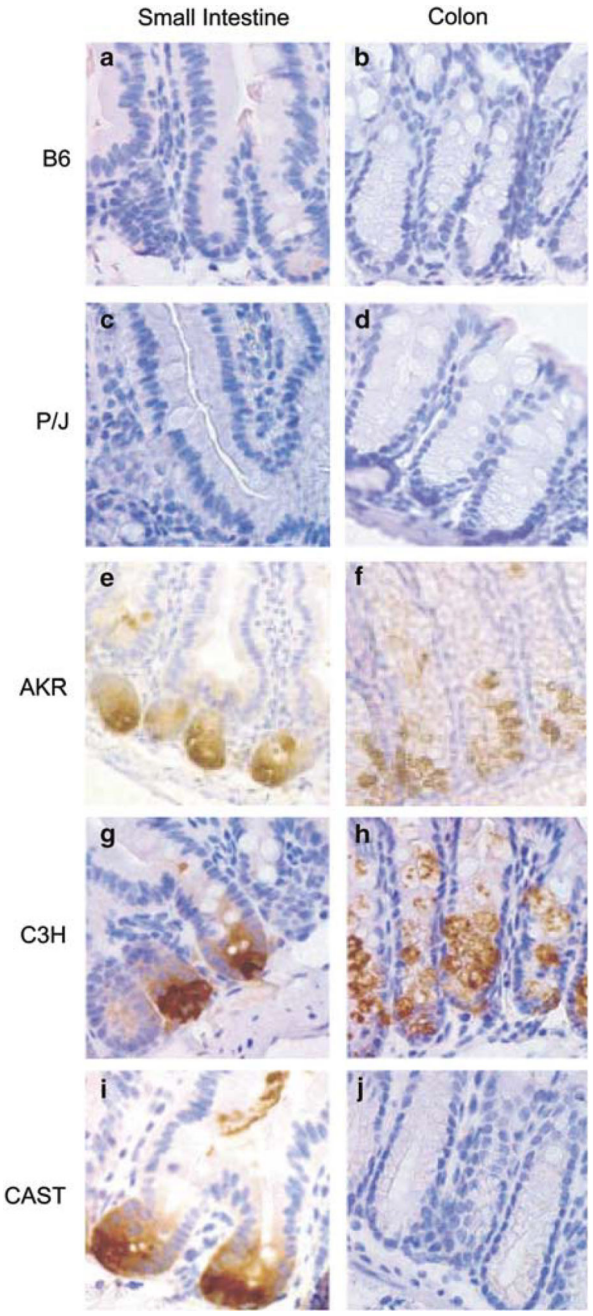

Figure 6.

Presence of $sPLA_2$ protein in the small intestine and colon of inbred mouse strains. Immunohistological staining with an $sPLA_2$ antibody (see Materials and methods) of tissue sections from the small intestine (**a, c, e, g, i**) and colon (**b, d, f, h, j**) of adult C57BL/6J (**a** and **b**), P/J (**c** and **d**), AKR (**e** and **f**), C3H/HeJ (**g** and **h**), and CAST/Ei (**i** and **j**) mice. Positive staining with the $sPLA_2$ antibody appears brown. B6 and P/J mice show no detectable staining with the $sPLA_2$ antibody in the small intestine and colon, consistent with both strains being null for *$sPLA_2$-IIA* (MacPhee *et al.*, 1995). C3H and CAST mice show intense cytoplasmic staining for $sPLA_2$ at the base of small intestinal crypts, consistent with

both strains being wildtype for *sPLA$_2$-IIA* (MacPhee *et al.*, 1995; Koratkar *et al.*, 2002). This pattern of expression is consistent with previously published reports (Mulherkar *et al.*, 1993). C3H mice show intense staining in Goblet cells of the colon, whereas CAST mice show much lighter, but positive, staining in Goblet cells of the colon

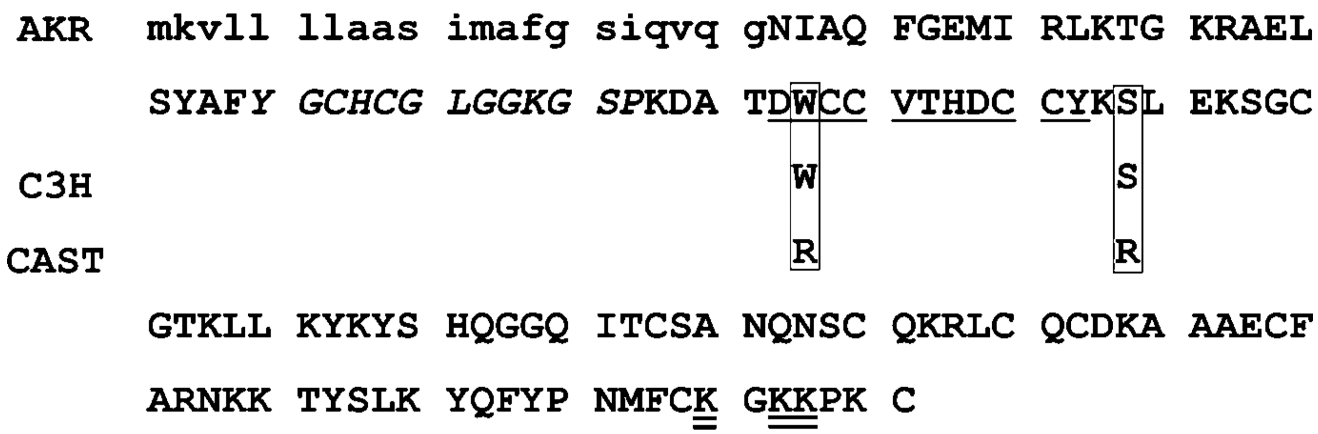

Figure 7.

Sequence of the *sPLA$_2$-IIA* gene in the inbred mouse strains AKR/J, C3H/HeJ and CAST/Ei. The coding region of the *sPLA$_2$-IIA* gene is shown. Lowercase letters indicate the signal peptide (Six and Dennis, 2000). Underlined letters indicate the active site for phospholipid cleavage (Valentin and Lambeau, 2000). Italicized letters indicate the calcium binding loop (Valentin and Lambeau, 2000). Double-underlined letters indicate heparin binding sites (Murakami *et al.*, 1996; Kirtane and Mulherkar, 2002). Rectangular boxes indicate amino-acid differences. The CAST strain has two arginines (at codons 63 and 74) that are conserved in the human genome (www.ensembl.org/Homo_sapiens/). The AKR and C3H strains differ from CAST at nucleotide 187 (C to T), resulting in a tryptophan at codon 63 instead of an arginine; note that this change occurs within the active site for phospholipid cleavage. The AKR and C3H strains also differ from CAST at nucleotide 220 (C to A), resulting in a serine at codon 74 instead of an arginine; this change occurs one base pair downstream of the active site, in a conserved domain that has bactericidal properties (Koduri *et al.*, 2002)

Table 1

Small intestinal polyp multiplicity and colon polyp incidence in *Apc*$^{Min/+}$ mice

Strain[a]	Mom1 locus[b]	Number of mice	Small intestine average polyps number[c]	Colon polyp incidence[d]	Days of age
B6 *Apc*$^{Min/+}$	*S/S*	28	63±25	68%	185–215[e]
(P/J × B6 *Apc*$^{Min/+}$)F1	*S/S*	23	31±12	9%	120–180
(C3H × B6 *Apc*$^{Min/+}$)F1	*R/S*	10	8±5	0%	120–150
(CAST × B6 *Apc*$^{Min/+}$)F1	*R/S*	11	7±4	0%	185–215[e]

[a] Comparisons of average small intestinal polyp multiplicity and colon polyp incidence in *Apc*$^{Min/+}$ F1 offspring from crosses of wildtype B6, P/J, C3H and CAST females with B6 *Apc*$^{Min/+}$ males.

[b] The *Mom1* locus is represented as 'S' for susceptible or 'R' for resistant.

[c] Polyp numbers obtained from the small intestine of each group are represented as mean±s.d.

[d] Colon polyp incidence is represented as the percentage of mice with ≥1 colon polyp (number of mice with colon polyps/total number of mice × 100).

[e] Data from Koratkar *et al.*, 2004