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The *Mom1*^{AKR} intestinal tumor resistance region consists of *Pla2g2a* and a locus distal to *D4Mit64*

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The *Mom1* (*M*odifier *o*f *M*in-*1*) region of distal chromosome 4 was identified during a screen for polymorphic modifiers of intestinal tumorigenesis in *Apc*$^{Min/+}$ mice. Here, we demonstrate that the *Mom1*^{AKR} allele consists of two genetic components. These include the secretory phospholipase *Pla2g2a*, whose candidacy as a *Mom1* resistance modifier has now been tested with several transgenic lines. A second region, distal to *Pla2g2a*, has also been identified using fine structure recombinants. *Pla2g2a*^{AKR} transgenic mice demonstrate a modest resistance to tumorigenesis in the small intestine and a very robust resistance in the large intestine. Moreover, the tumor resistance in the colon of *Pla2g2a*^{AKR} animals is dosage-dependent, a finding that is consistent with our observation that Pla2g2a is expressed in goblet cells. By contrast, mice carrying the distal *Mom1* modifier demonstrate a modest tumor resistance that is confined to the small intestine. Thus, the phenotypes of these two modifier loci are complementary, both in their quantitative and regional effects. The additive effects and tight linkage of these modifiers may have been necessary for the initial identification of the *Mom1* region. *Oncogene* (2000) **19,** 3182–3192.

Keywords: *Mom1*; tumor resistance; *Apc*; Min mouse; goblet cell; *Pla2g2a*

Introduction

Apc$^{Min/+}$ mice carry a germline mutation in the adenomatous polyposis coli (*Apc*) gene (Su *et al*., 1992; Shoemaker *et al*., 1997; Gould and Dove, 1998). These animals have proven to be valuable models for studying genes that quantitatively affect the development of human colon cancer (Moser *et al*., 1990; Shoemaker *et al*., 1997; Gould and Dove, 1998). Similar germline mutations in the homologous human *APC* gene lead to familial adenomatous polyposis (Groden *et al*., 1991). Studies of *Min* on different genetic backgrounds have led to the identification of novel modifier loci (Moser *et al*., 1992; Dietrich *et al*., 1993; Gould *et al*., 1996b), while combinations of *Min* with other mutant strains have helped to elucidate the role in tumorigenesis of previously identified genes, such as *Dnmt1* (Laird *et al*., 1995; Cormier and Dove, in press) and *p53* (Halberg *et al*., 2000). The search for the first polymorphic *Min* modifier locus was prompted by the observation that progeny of (AKR × B6) F1 crosses developed only 20% as many intestinal adenomas as B6-Min control animals, indicating that the AKR strain carried one or more dominant resistance modifiers (Moser *et al*., 1992). An (AKR × B6) F1 × B6-Min backcross was employed to map a locus, *M*odifier *o*f *M*in-1 (*Mom1*), to a 15-cM region on distal chromosome 4. The genotype at *Mom1* explained about 50% of the reduction in tumor multiplicity observed in (AKR × B6) F1 mice (Dietrich *et al*., 1993). A *Mom1* congenic strain was bred in which a 35-cM region of AKR distal chromosome 4 carrying *Mom1* was introduced onto the B6 genetic background (Gould *et al*., 1996a). *Mom1* congenic heterozygotes that carried *Min* (B6-Min *Mom1*$^{AKR/B6}$) developed only 50% as many intestinal adenomas as B6-Min control siblings, whereas *Mom1* congenic homozygotes that carried *Min* (B6-Min *Mom1*$^{AKR/AKR}$) developed only 25% as many tumors, demonstrating that this modifier acts semi-dominantly (Gould *et al*., 1996a). *Mom1*^{AKR} was further shown to reduce net tumor growth rate (Gould *et al*., 1996a), and probably to act non-cell autonomously, although within the crypt cell lineage (Gould *et al*., 1996c).

MacPhee and colleagues (1995) proposed *Pla2g2a*, the calcium-dependent non-pancreatic secretory phospholipase (Dennis, 1994), as a candidate gene for *Mom1*. *Pla2g2a* maps to a region of distal mouse chromosome 4 that shares conserved linkage with human chromosome 1p35-36 (Tischfield *et al*., 1996). This candidacy was supported by concordance between *Pla2g2a* genotype and *Mom1* phenotype in nine inbred strains that had been crossed to B6-Min, and subsequently backcrossed to B6 (Gould *et al*., 1996b). *Pla2g2a* was found to have a frameshift mutation in three strains (B6, 129, and BTBR) that have a *Mom1* allele that confers sensitivity to tumorigenesis, while *Pla2g2a* is wildtype in six strains (AKR, BALB, SWR, DBA, MA, and CAST) that carry a *Mom1* resistance allele (MacPhee *et al*., 1995; Kennedy *et al*., 1995; Gould *et al*., 1996b). However, another interesting candidate gene, *Rap1Gap*, also maps to the *Mom1* region (Gould *et al*., 1996b). Fine-structure recombinant mapping ultimately positioned *Mom1* to a 4-cM interval that included *Pla2g2a*, but excluded *Rap1Gap*, on the basis of a single recombinant line (Gould *et al*.,

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1996b). Interestingly, recombinant analysis further suggested that *Mom1* may consist of more than one component: two recombinant lines carrying wildtype *Pla2g2a*, but missing a distal section of the *Mom1* region, demonstrated a partial resistance that was quantitatively intermediate between that of B6-Min and the congenic B6-Min $Mom1^{AKR/B6}$ (Gould *et al*., 1996b).

To test the hypothesis that *Pla2g2a* is responsible for part or all of the *Mom1* phenotype, we analysed a transgenic line carrying the functional $Pla2g2a^{AKR}$ allele on a B6 background (Cormier *et al*., 1997). Overexpression of *Pla2g2a* reduced intestinal adenoma multiplicity by 50%, similar to congenic B6-Min $Mom1^{AKR/B6}$ mice. However, the $Pla2g2a^{AKR}$ transgene did not add to the resistance phenotype of $Mom1^{AKR/B6}$ congenic mice. This observation was inconsistent with the known semi-dominant nature of *Mom1*'s tumor resistance phenotype. This inconsistency may have resulted from unknown factors affecting the temporal and spatial regulation of transgene expression. Alternatively, the second component of *Mom1*, suggested by the studies of Gould *et al*. (1996b), may have been lacking. To test the latter hypothesis and to extend our previous work, we have tested congenic recombinant lines that further separate the *Mom1* interval into discrete regions whose recombinant breakpoints lie immediately distal to *Pla2g2a*. In addition, we have generated two more $Pla2g2a^{AKR}$ transgenic lines ($Pla2g2a^{948}$ and $Pla2g2a^{959}$), and examined the phenotypes of a larger set of test animals from the original transgenic line ($Pla2g2a^{KH1}$).

		Adenoma counts, mean ±SD			
Region		*Line 57 (N=8; B6 N=8)*	*Line 29 (N=13; B6 N=8)*	*Line 63 (N=8; B6 N=8)*	*Congenic Line (N=8)*
Total	Line	92 ± 29	86 ± 28	73 ± 32	59 ± 11
	B6	123 ± 30	144 ± 39	132 ± 31	
		p = 0.014	p = 0.001	p = 0.002	
Proximal	Line	8.0 ± 2.5	6.6 ± 2.7	5.0 ± 2.2	7.0 ± 2.3
	B6	7.8 ± 1.6	9.0 ± 3.2	9.3 ± 3.6	
		p = 0.46	p = 0.077	p = 0.008	
Medial	Line	9.0 ± 4.0	9.4 ± 4.9	9.4 ± 8.7	6.3 ± 3.2
	B6	14 ± 5.3	16 ± 4.5	16 ± 9.7	
		p = 0.020	p = 0.002	p = 0.057	
Distal	Line	7.3 ± 2.4	5.5 ± 3.3	6.0 ± 5.2	4.6 ± 3.5
	B6	7.1 ± 5.0	9.0 ± 2.6	7.1 ± 3.6	
		p = 0.48	p = 0.01	p = 0.23	
Colon	Line	4.9 ± 5.0	2.0 ± 1.8	2.3 ± 2.5	0.9 ± 0.8
	B6	2.9 ± 2.8	4.4 ± 1.8	7.1 ± 5.0	
		p = 0.14	p = 0.008	p = 0.01	

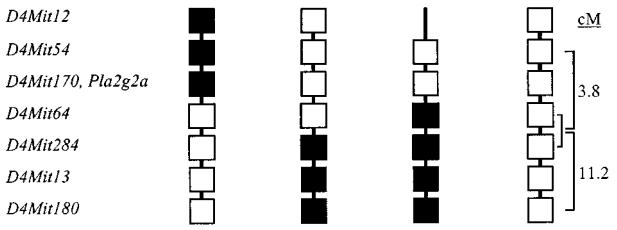

Figure 1 A map of the *Mom1* region comparing the region defined by the congenic $Mom1^{AKR}$ region and various B6-$Mom1^{AKR}$ recombinant lines (29, 63, and 57), that divide the *Mom1* locus near the marker *D4Mit64*. Following crosses to B6-Min mice, Min progeny from these lines were sacrificed at about 94 days, and their overall and regional tumor multiplicities were determined. Lines 57, 63, and 29 were significantly different than B6-Min control siblings and *Mom1* congenics in tumor multiplicity ($P<0.05$). Congenic line represents $Mom1^{AKR/B6}$ age-matched class. *Pla2g2a* maps to ~68 cM. AKR = □, B6 = ■

Results

Recombinant lines divide the Mom1 *region near D4Mit64 into two complementary resistance loci*

Previous phenotyping of fine-structure recombinants in the *Mom1* region revealed that B6-Min mice carrying the AKR allele of *Pla2g2a* are resistant to intestinal tumorigenesis (Gould *et al*., 1996b). Notably, B6-$Mom1^{AKR}$-derived recombinant line 25 and its derivative line 32, which carry a $Pla2g2a^{AKR}$ allele, were only partially resistant when compared with the phenotype of heterozygous B6-Min $Mom1^{AKR}$ congenic mice. To determine whether an AKR allele of a locus distal to *Pla2g2a* (which we find to be recombinationally inseparable from *D4Mit170*) might confer the remainder of the *Mom1* resistance phenotype, we tested the phenotype of recombinant line 57, whose breakpoint lies immediately distal to *Pla2g2a*, in the interval between *D4Mit170* and *D4Mit64*. The AKR fragment carried in line 57 extends through the marker *D4Mit180*, potentially as far as the telomere at about 84-cM. Concurrently, we tested the phenotypes of lines 29 and 63, which, like recombinant line 25, carry a proximal $Mom1^{AKR}$ segment that includes an AKR-derived *Pla2g2a* (Figure 1).

Heterozygous female recombinant mice were crossed to B6-Min males, and tumors were counted and measured along the intestinal tract of all Min progeny. Mice from recombinant line 29 developed significantly fewer tumors than B6-Min control siblings ($P=0.001$), but they also developed significantly more tumors than an age-matched class of B6-Min $Mom1^{AKR/B6}$ congenic mice ($P<0.05$). Thus, recombinant line 29 demonstrated an intermediate tumor resistance phenotype. Recombinant line 63, which carries a smaller proximal AKR region than line 29 (Figure 1), also displayed a phenotype that was significantly different from that of B6-Min control mice and that was marginally different (approaching statistical significance at $P=0.08$) from $Mom1^{AKR}$ congenics. These data indicate that the region of AKR carried by lines 29 and 63 confers partial *Mom1* resistance to intestinal tumorigenesis.

Min animals carrying the line 57 recombinant chromosome also display partial *Mom1* resistance (see Figure 1). Line 57 mice develop significantly fewer tumors than B6-Min siblings ($P=0.01$), but develop significantly more than $Mom1^{AKR/B6}$ congenics ($P=0.004$). Thus, the proximal and distal AKR resistance alleles appear to be complementary, together approximating the resistance conferred by the entire AKR region present in an age-matched class of $Mom1^{AKR/B6}$ congenics. For example, the 40% reduction in intestinal tumor multiplicity observed in recombinant line 29 and the 25% reduction imparted by recombinant line 57, together approximate the 55–60% reduction in tumor multiplicity seen, both currently and historically, in congenic mice heterozygous for the $Mom1^{AKR}$ resistance allele.

Strikingly, the proximal and distal AKR resistance alleles differ with respect to their effects on different regions of the intestine. Tumor counts taken from 4-cm sections from the small intestine (proximal, medial, and distal) and from the entire colon revealed that recombinant lines 63 and 29 (like *Mom1*^{AKR} congenics) reduce tumorigenesis throughout the intestinal tract (Figure 1). By contrast, the effect of recombinant line 57 was observed only in the medial 4-cm region. This medial region lies adjacent to a tumor cluster region that extends towards the distal area of the small intestine (K Haigis, unpublished results). Thus, it is likely that line 57 affects not only the medial 4-cm region tested, but the tumor cluster region as well.

The partial resistance observed is seen in tumor size as well as number: recombinant lines 29, 57, (see Table 1) and 63 (data not shown), like *Mom1*^{AKR} congenics, showed a reduction in the average maximum diameter of adenomas measured in the small intestine at 90 days of age. This effect was stronger in the proximal than in the distal recombinant lines.

Tumor resistance phenotypes of Pla2g2a^{AKR} *transgenic mice are similar to recombinant lines 63 and 29*

We have studied the intestinal tumor phenotypes of three transgenic lines that carry a 41-kb DNA fragment from AKR that encodes a functional copy of the *Pla2g2a* gene (Cormier *et al*., 1997). Min-*Pla2g2a*^{AKR} transgenic lines reduce tumor multiplicity (Table 2) to an extent similar to recombinant lines 63 and 29 (see Figure 1), which carry an AKR-derived copy of *Pla2g2a*. The tumor resistance effects of lines *Pla2g2a*^{KH1} (−28%), *Pla2g2a*^{948} (−34%), and *Pla2-g2a*^{959} (−24%) resemble the 40∼45% reduction observed in line 29 and 63. These reductions occurred throughout the intestinal tract (e.g., see the tumor distribution of *Pla2g2a*^{KH1} in Table 3), as in recombinant lines 63 and 29, and *Mom1*^{AKR} congenics, but in contrast to recombinant line 57.

The tumor resistance demonstrated in *Pla2g2a*^{AKR} transgenic lines appears to correspond to the level of Pla2g2a expression. Normalized to Pla2g2a expression in *Mom1*$^{AKR/AKR}$ congenics, protein levels in the small intestine of *Pla2g2a*^{AKR} transgenics were found to be less than *Mom1*$^{AKR/AKR}$ congenics (line 959 = 70%, line 948 = 70%, line KH1 = 50%). Pla2g2a protein expression correlates with the modest small intestine resistance phenotype of these transgenic lines. In the large intestine Pla2g2a transgenic expression is far more robust (line 959 = 260%, line 948 = 570%, line KH1 = 290%). Transgenic line *Pla2g2a*^{948}, the strongest Pla2g2a expressor in the large intestine, reduces colon tumor incidence by a factor greater than 10 (see Figures 2, 3 and Table 2).

The *Pla2g2a*^{AKR} transgenics each confer a reduction in the size of tumors in the small intestine of mice as measured at 90 days of age (see Table 1). *Pla2g2a*^{AKR} transgenics also appear to reduce the size of colonic adenomas, with the strongest effect in line 948 (data not shown). Transgenic line *Pla2g2a*^{KH1}, like *Mom1*^{AKR} (Gould *et al*., 1996a), also slows the net growth rate of tumors in the small intestine, especially in the period between 90 and 120 days (data not shown). We note that, among these different mouse lines, *Mom1*^{AKR} congenics have the strongest effect on tumor growth rate (R Cormier, unpublished results), consistent with the hypothesis that *Mom1* has more than one component.

Table 1 Analysis of average maximal tumor size (in the small intestine) in recombinant lines 57 and 29, *Pla2g2a*^{AKR} transgenics, and *Mom1*$^{AKR/B6}$ congenics

Class	n	*Average maximal size* (mm)	
Rec-29	13	1.12	$P = 0.02$
B6-Min	8	1.47	
Rec-57	8	1.40	$P = 0.12$
B6-Min	8	1.55	
Tg-KH1	41	1.21	$P = 0.06$
B6-Min	46	1.28	
Tg-948	13	1.21	$P = 0.10$
B6-Min	11	1.31	
Tg-959	26	1.17	$P = 0.005$
B6-Min	29	1.31	
Mom1$^{AKR/B6}$	8	1.04	

Comparison of tumor size in the small intestine of recombinant lines 29, 57, *Pla2g2a*^{AKR} transgenic lines (KH1, 948, 959), and *Mom1*^{AKR} congenic heterozygotes. Mice were sacrificed at 90 days (transgenic experiment) or 94 days (recombinant experiment) of age, and the average maximum diameters of tumors in the small intestine were measured. Note that *Pla2g2a*^{AKR} also appears to reduce adenoma size in the large intestine in a dose-dependent fashion, but owing to the difficulty of accurately measuring the diameters of the pedunculated colonic tumors, we have excluded these from our analysis. One-sided *P* values represent comparison of each group with age-matched B6-Min controls, often siblings. Studies utilizing recombinant lines 29, and 57 and *Mom1*^{AKR} congenics were scored by one observer (AJ Lillich), while analyses of *Pla2g2a*^{AKR} transgenic lines KH1, 948 and 959 were scored by a different observer (RT Cormier), in a separate experiment. *n* = number of mice

Table 2 Effect of *Pla2g2a*^{AKR} transgene on intestinal tumor multiplicity

Class	n	*Total*	*Colon*
Tg-KH1	69	67	1.1
Mom1$^{AKR/B6}$	71	42	1.3
Tg-KH1 & *Mom1*$^{AKR/B6}$	64	40	0.9
B6-Min control	67	91	3.2
Tg-948	73	74	0.7
B6-Min control	58	112	7.5
Tg-959	118	81	1.8
B6-Min control	129	107	6.5

Pla2g2a^{AKR} transgenic lines show strong tumor resistance in the large intestine and an intermediate but significant effect in the small intestine. Following crosses to B6-Min, Min transgenic progeny were sacrificed at 90 days of age, and tumors were counted from the entire intestinal tract. All three transgenic lines were significantly different from B6-Min non-transgenic littermates ($P < 0.05$). *n* = number of mice

Table 3 Effect of *Pla2g2a*^{AKR} transgene on tumor distribution in small intestine

Class	*Proximal*	*Medial*	*Distal*
Tg-KH1	4.4	8.4	4.7
Mom1$^{AKR/B6}$	3.4	5.3	3.4
KH1 & *Mom1*$^{AKR/B6}$	3.1	5.4	2.8
B6-Min control	6.0	12.3	7.9

Transgenic line *Pla2g2a*^{KH1} shows tumor resistance throughout the intestinal tract. Min transgenic mice were sacrificed at 90 days of age, and their tumor multiplicities were counted from 4-cm regions from the proximal, medial, and distal small intestine

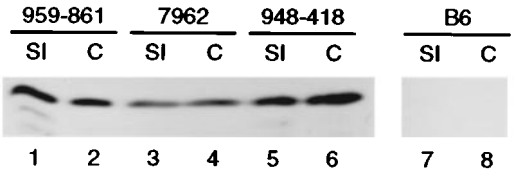

Figure 2 Level of Pla2g2a protein measured in the small intestine and colon. Acid-soluble protein extracts were prepared from the small intestine (SI) and colon (C) of B6 transgenic mice *Pla2g2a*^{959} (lanes 1–2), *Pla2g2a*^{KH1} (lanes 3–4), or *Pla2g2a*^{948} (lanes 5–6), (20 μg) and a B6 control (lanes 7–8), (40 μg). These extracts were subjected to Western blot analysis with Pla2g2a antiserum. *Pla2g2a*^{KH1} is a positive control for Pla2g2a immunoreactivity (Cormier *et al*., 1997); B6 mice are a negative control, since they are naturally mutant for *Pla2g2a*. Note that the lanes 1–6 were exposed for 15 s while lanes 7–8 were exposed for 5 min. Signals were quantitated and normalized to *Mom1*$^{AKR/AKR}$ (band not shown). All signals were within a linear range of intensity. Relative amount of pixel intensity (ratio of each lane over *Mom1*$^{AKR/AKR}$ signal): lane 1 (0.7), lane 2 (2.6), lane 3 (0.5), lane 4 (2.9), lane 5 (0.7), lane 6 (5.7), lane 7 (0), lane 8 (0). Western blot analysis was also performed on tissues from recombinant lines 29, 57, and 63, and *Mom1*^{AKR} congenics (data not shown). Lines 29 and 63 showed Pla2g2a detection similar to that of *Mom1*^{AKR} congenic line, with overall amounts of signal estimated to be somewhat below that of *Pla2g2a*^{KH1}, and significantly below *Pla2g2a*^{948} in the large intestine. Consistent with PCR analysis, line 57 was similar to B6 mice and had no signal corresponding to the approximately 14 kDa Pla2g2a protein

Pla2g2a is expressed by the goblet cells in the large intestine

We have determined that goblet cells are the source of Pla2g2a produced in the large intestine, while Pla2g2a is expressed by both goblet and Paneth cells in the small intestinal crypt (see Figures 3 and 4). In the large intestine, immunostaining for Pla2g2a is detected only in the interior of the crypts, generally staining the mucinous, goblet-cell region facing the crypt lumen. The strong over-expression of Pla2g2a in goblet cells of line *Pla2g2a*^{948} is illustrated in Figure 3a,b. Consistent with Western blot analysis (Figure 2) weaker immunostaining in the large intestine was detected in transgenic lines *Pla2g2a*^{959} (Figure 3c) and *Pla2g2a*^{KH1} (Figure 3d). B6 large intestine, mutant for *Pla2g2a*, is shown in Figure 3e,f. Interestingly, while we cannot detect Pla2g2a signal in Western blots (Figure 2), we have consistently observed a very faint immunostaining for Pla2g2a (above the minus-antibody control), which may be recognizing other type II-Pla2s (such as Pla2g2d) which are expressed at low levels in the mouse intestine.

The goblet cell-specific spatial expression pattern of Pla2g2a was confirmed in serial sections obtained from transgenic line *Pla2g2a*^{948} that were stained for alcian blue, which detects the acidic mucopolysaccharides of goblet cells (Figure 4). Enterocytes of the intercrypt table facing the main intestinal lumen do not stain positively for either Pla2g2a or alcian blue (Figure 4c,d). In the large intestine, goblet cell expression by Pla2g2a is very apparent by direct comparison of serial sections stained for either alcian blue or Pla2g2a Ab (Figure 4a–d). There is nearly complete concordance in alcian blue and Pla2g2a staining profiles. We can also detect Pla2g2a immunostaining and alcian blue staining in the small intestine (Figure 4e,f).

The finding that Pla2g2a is expressed by the goblet cell population may shed light on the phenotypes seen in *Pla2g2a*^{AKR} transgenic lines and in proximal region recombinant lines. Goblet cells represent only ~5–10% of epithelial cells in the small intestine but more than 60% in the large intestine (Paulus *et al*., 1993; Itoh *et al*., 1999). The *Pla2g2a* transgene confers only a modest tumor resistance in the small intestine, but a far more robust response in the large intestine. These regional differences may be attributable to the relative distribution of goblet cells in the different regions of the intestine.

Pla2g2a^{AKR} *transgenic mice develop normally*

Pla2g2a^{AKR} mice appear phenotypically identical to non-transgenic littermates. *Pla2g2a* transgenic mice do not develop any of the abnormalities reported in mice expressing a human *PLA2G2A* transgene (Grass *et al*., 1996; Nevalainen *et al*., 1997), such as epidermal and adnexal hyperplasia, hyperkeratosis, alopecia, and juvenile cachexia. This finding may not be surprising since mPla2g2a and hPLA2G2A may differ in some physiological functions, as suggested recently by Lambeau and colleagues (Cupillard *et al*., 1999). These differences may extend to intestinal functions and therefore account for some of the difficulty in reconciling mPla2g2a's tumor resistance with some of the known actions of hPLA2G2A (discussed further below).

The intestine of transgenic mice also appears identical to non-transgenic siblings, with no observed differences in tissue architecture, length, or intestinal crypt height. Transgenic line *Pla2g2a*^{KH1} does appear to have an increased frequency of microphthalmia and anophthalmia (R Cormier and K Hong, unpublished observations), but this phenotype was not observed in lines *Pla2g2a*^{948} or *Pla2g2a*^{959}, which express the transgene at equivalent or higher levels in the intestine. This suggests that the *Pla2g2a*^{KH1} eye phenotype may be specific to either its genomic insertion site or its regulation in cells of the eye lineage.

Discussion

Mom1 *is a complex locus*

Our functional studies of the *Mom1* region illustrate some of the challenges inherent in the identification of quantitative trait loci (QTLs). While the use of inbred mice permits the generation of large mapping populations, current methodologies provide only limited resolution, even with the increasing availability of genetic markers. One is still left with the task of sifting through a fairly large and diverse set of candidate genes even in a relatively small region such as the *Mom1* locus (discussed by Dragani and Manenti, 1997). This difficulty is compounded when the QTL consists of multiple polymorphic modifiers, as we have shown here for *Mom1*. Ultimately, suitable candidates require functional genetic tests such as targeted knockouts, knockins, or the addition of transgenic DNA fragments.

We have generated three transgenic lines to test the function of *Pla2g2a*^{AKR}, expressed on a B6 background.

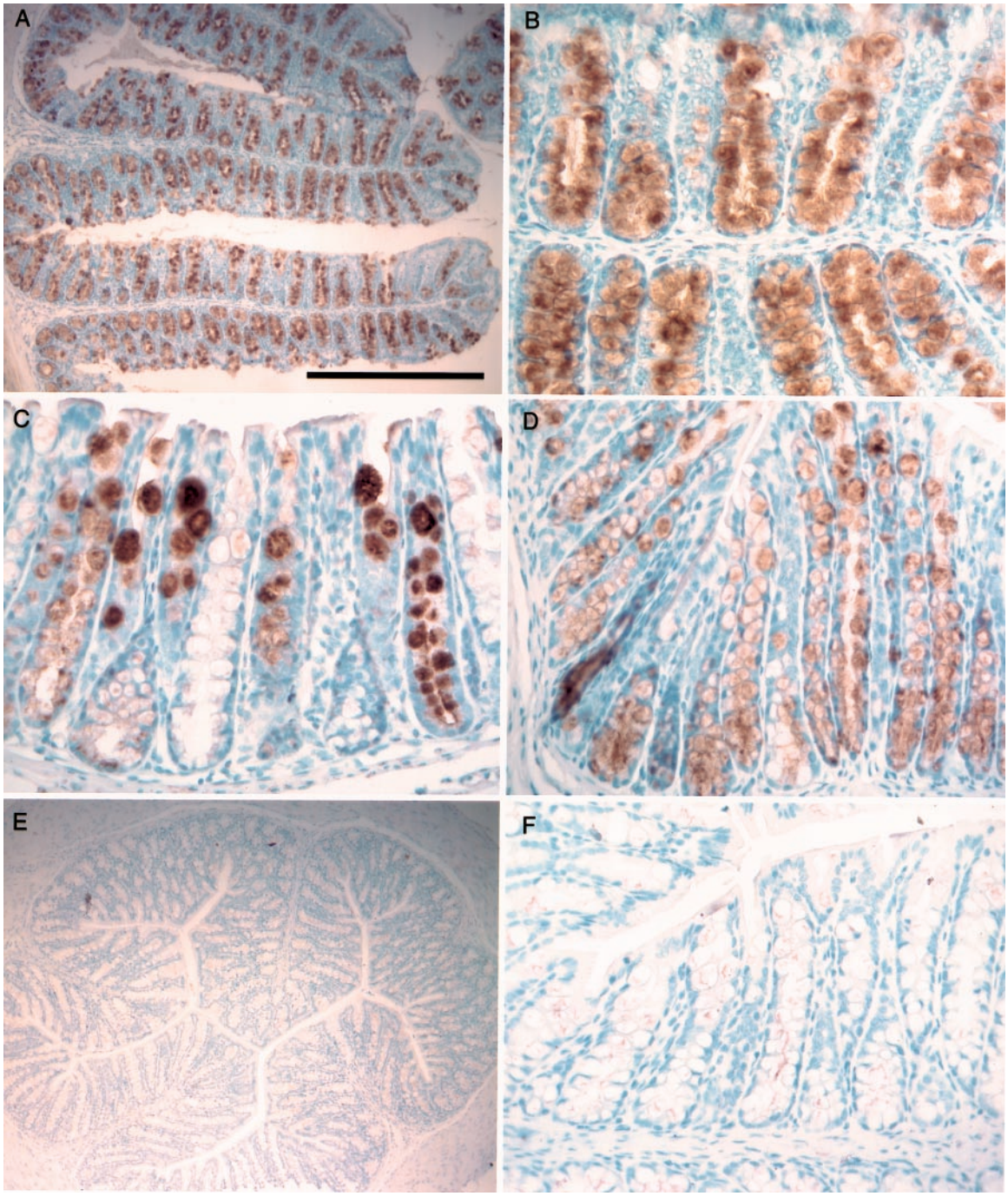

Figure 3 Spatial distribution and intensity of Pla2g2a protein in the large intestine. (**a**) *Pla2g2a*^{948}, an overview of the expression pattern in the large intestine, 10×. (**b**) Same view at 40×, with pronounced expression of Pla2g2a apparent in the intestinal crypt, in a mucinous region rich in goblet cells. (**c**) *Pla2g2a*^{959}, expression patterns at 40×. (**d**) *Pla2g2a*^{KH1}, 40×. (**e**) B6-*Apc*-wildtype age-matched large intestinal overview, 10×. (**f**) B6-*Apc*-wildtype age-matched mouse, 40×. Size bar = 0.5 mm

The phenotypes of these three lines provide clear genetic evidence that *Pla2g2a* provides active resistance to intestinal tumorigenesis in the mouse and accounts for a part of the *Mom1* phenotype. This resistance is robust in the large intestine, with Pla2g2a appearing to provide all of the *Mom1* effect in the colon. For example, transgenic line *Pla2g2a*^{948} reduced colon tumor multiplicity by a factor greater than 10. However, in the small intestine, Pla2g2a expression consistently provides only a modest reduction in tumor multiplicity (~30%). The phenotypes of proximal AKR region recombinant lines 29 and 63 resemble the phenotypes of the transgenic lines in having a broad intestinal tissue specificity and in displaying a resistance that is quantitatively intermediate between that of the B6-Min and B6-Min *Mom1*$^{AKR/B6}$ classes.

This analysis is also consistent with the phenotypes of recombinant lines 25 and 32, reported by Gould *et al*. (1996b) to display intermediate resistance. Lines 25, 32, and 29 all carry an AKR region from chromosome 4 defined by the markers *D4Mit12* to *D4Mit64*. We note that a previous analysis of recombinant line 29 did not reveal a statistically significant intermediate phenotype (Gould *et al*., 1996b). In that study, line 29 developed a number of tumors in the standard four intestinal regions sampled that was statistically indis-

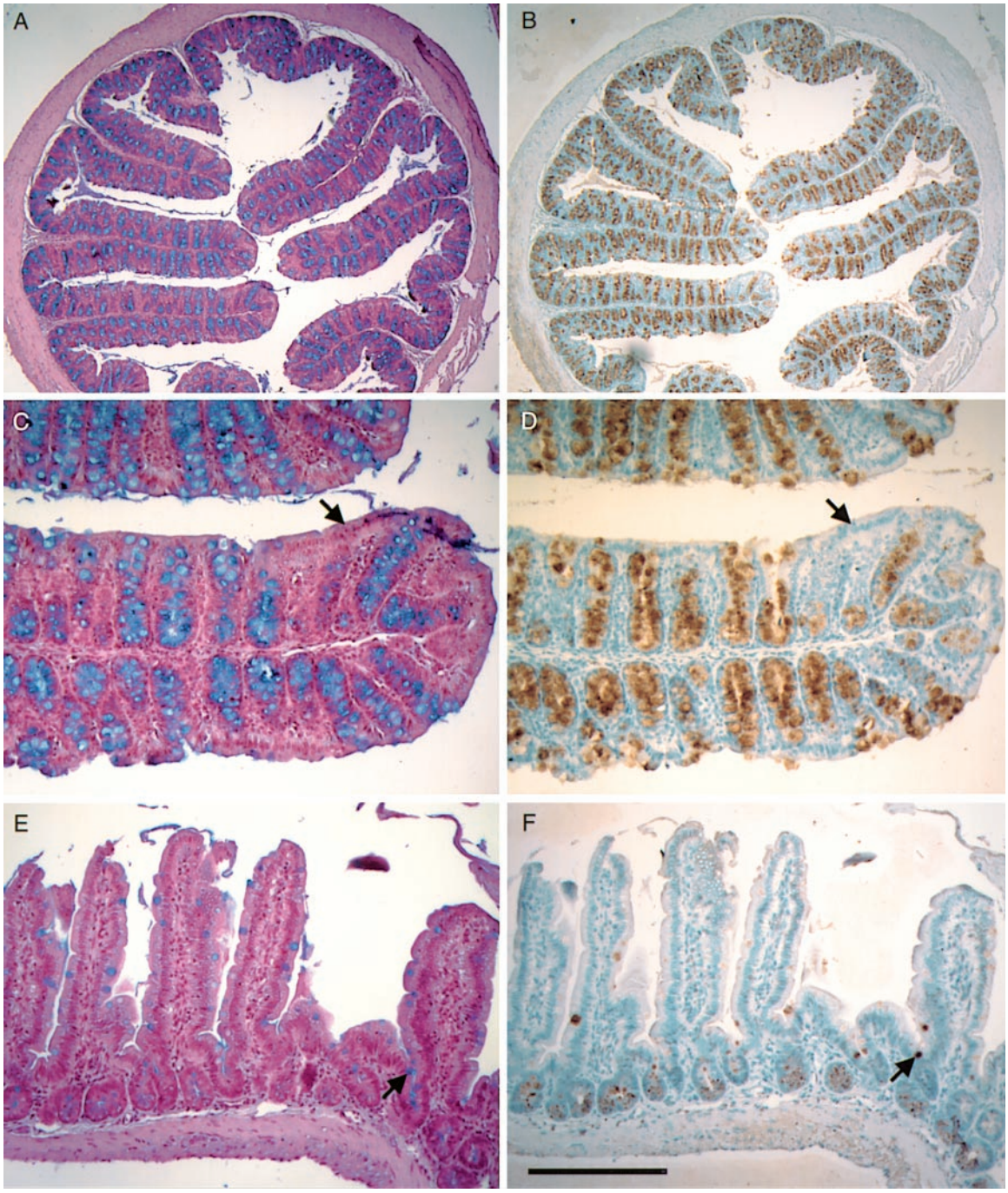

Figure 4 Spatial identity of cells expressing Pla2g2a and staining for alcian blue. Intestinal tissue sections immediately adjacent to those immunostained for Pla2g2a were stained with alcian blue, pH 2.5, to detect acidic mucopolysaccharides expressed by goblet cells. All sections were taken from a *Pla2g2a*^{948} transgenic mouse. (**a**) An overview of alcian blue staining in the large intestine, 5×. (**b**) Pla2g2a immunostaining, serial section, 5×. (**c**) Close-up of an intestinal fold depicted in A, stained for alcian blue. Note lack of staining in the intercrypt table region (see arrow), 20×. (**d**) Pla2g2a immunostaining, serial section; again note lack of expression in the intercrypt table region (arrow), 20×. (**e**) Small intestine, alcian blue staining. Note the random staining pattern consistent with a ~5–10% dispersed population of goblet cells in the small intestine, 10×. (**f**) Pla2g2a immunostaining, serial section; note the punctate staining in the Paneth cell region. Pla2g2a was also detected in random goblet cells (see arrow), 10×. Size bar = 0.2 mm

tinguishable from the number developed by the *Mom1*^{AKR} congenic line. In the re-analysis of multiple recombinant lines reported here, the intermediate phenotype of line 29 may have reached statistical significance because the entire intestine was scored and a larger total number of tumors was registered.

Analysis of a fine-structure recombinant (line 57), whose breakpoint lies immediately distal to *D4Mit170*, indicates that the complementary partner, or partners, for *Pla2g2a* in the small intestine lie in this distal *Mom1* region, probably mapping distal of *D4Mit64*. Our data suggest that this distal recombinant line provides tumor resistance that is restricted to a defined region of the small intestine, in an area that is likely to extend from the medial section into the adjacent tumor cluster site. This restriction observed in line 57's effect is unlike the broad range of action observed in the proximal recombinant lines, *Pla2g2a*^{AKR} transgenic lines, or *Mom1*^{AKR} congenics.

Line 52, which carries an AKR fragment whose breakpoint lies distal of *D4Mit64*, supports the identification of a second *Mom1* component and suggests that it lies at least 2-cM from *Pla2g2a*. This line resembles line 57 in conferring a modest resistance that is confined to the medial region of the small intestine (data not shown). This effect is only suggestive, however, ($P=0.095$). Considered together, the data from lines 57 and 52 and the fact that proximal line 29's breakpoint is also distal to *D4Mit64* suggest that the distal *Mom1* modifier lies between *D4Mit64* and the telomere.

Parenthetically, we note that the phenotype of *Pla2g2a*^{KH1} transgenic mice varies from that previously reported by our group (Cormier *et al*., 1997). Tumor multiplicities from a much larger set of 69 transgenic mice demonstrated only a 28% reduction in overall tumor number, which is significantly different from our previous report of a 50% reduction in tumors, scored from an initial set of 10 transgenic mice. One potential explanation for this variance is a 'litter effect' that we have observed in *Min* tumor phenotypes. Severe or attenuated tumor phenotypes often cluster within litters, a finding that may necessitate larger sample sizes. Notably, the 28% reduction in tumors observed in the larger set of KH1 transgenic mice is similar to the phenotypes of lines 948 (−34%) and line 959 (−24%).

Pla2g2a, lipid metabolism, and intestinal cancer

The nomination of *Pla2g2a* as a negative regulator of tumorigenesis in the intestine has been controversial. With a single exception (Nimmrich *et al*., 1997), loss of *Pla2g2a* has not been observed in human colon cancer (Riggins *et al*., 1995; Praml *et al*., 1998; Dobbie *et al*., 1997; Spirio *et al*., 1996), although as noted by Dove *et al*. (1994), Gould *et al*. (1996a), and MacPhee *et al*. (1995), one would not expect to observe LOH for a secreted factor that presumably acts non-cell autonomously within the mouse intestinal crypt.

One function of Pla2g2a is to catalyze the enzymatic release of long-chain fatty acids, such as arachidonic acid, at the *sn-2* position of membrane glycerophospholipids. Arachidonic acid is a substrate for cyclooxygenases 1 and 2 (COX-1 and COX-2), the primary enzymes responsible for prostaglandin biosynthesis (Herschmann, 1996; Murakami *et al*., 1998, 1999; Vane *et al*., 1998; Fourcade *et al*., 1995; Balsinde *et al*., 1998; Tischfield, 1997). COX-2 has been implicated genetically (Oshima *et al*., 1996) and pharmacologically (Yang *et al*., 1998; Shiff *et al*., 1995; Piazza *et al*., 1995; Tsujii and Dubois, 1995; Tsujii *et al*., 1997; Beazer-Barclay *et al*., 1996; Mahmoud *et al*., 1998; Boolbol *et al*., 1996; Jacoby *et al*., 1996; Taketo, 1998; Rao *et al*., 1995; Quesada *et al*., 1998; Nakatsugi *et al*., 1997) in the enhancement of intestinal tumorigenesis in both humans and rodents. Further, COX-2 is reported to be upregulated in both human colon cancer and in *Min* adenomas (Williams *et al*., 1996). The designation of COX-2 as a positive tumor regulator has led to the proposal that other genes (such as *PLA2G2A*) and various eicosanoids in the COX-2 pathway might also positively impact the adenoma and thus should be considered appropriate therapeutic targets (Ikegami *et al*., 1998; Rao *et al*., 1996). However, accumulating data suggest that Pla2G2a and the prostaglandin biosynthetic pathway are not associated with COX-dependent tumorigenesis. Non-steroidal anti-inflammatory drugs (NSAIDs) that target COX-2 have been shown to induce regression of *Min* adenomas independent of prostaglandin biosynthesis (Chiu *et al*., 1997; Moorghen *et al*., 1998; Mahmoud *et al*., 1998). NSAIDs can also inhibit the proliferation of human colon cancer cells (Hanif *et al*., 1996; Erickson *et al*., 1999; McCracken *et al*., 1996; Levy, 1997), as well as chemically induced colon tumors in rats (Reddy *et al*., 1999; Charalambous *et al*., 1998), independent of prostaglandins. Crucially, the molecular genetic studies reported in this paper and its predecessors provide strong evidence that Pla2g2a diminishes, rather than enhances, intestinal tumorigenesis in the mouse, particularly in the large intestine, where this effect is dosage-dependent.

Pla2g2a: a role in mucosal barrier function?

Immunohistochemical analysis of Pla2g2a in the large intestine of transgenic mice shows that Pla2g2a is robustly expressed by the goblet cells (Figures 3 and 4). In the small intestine Pla2g2a is predominantly expressed by the Paneth cells that reside at the base of the crypt of Lieberkuhn (Ouellette, 1997; Mulherkar *et al*., 1993; Cormier *et al*., 1997). In this study we also detected Pla2g2a immunostaining in random goblet cells in the small intestine (Figure 4f). These observations are consistent with the hypothesis of Garabedian *et al*. (1997) that goblet and Paneth cells share a common precursor in the small intestine. These investigators found that residual Pla2g2a expression continued to be detected in the mouse small intestine after Paneth cell ablation.

In the large intestine, products of the goblet cells are thought to protect the mucosa from injury, possibly by preventing bacterial invasion that would prompt damaging host immune responses (Fukushima *et al*., 1999). Pla2g2a expression in the goblet cell population could help to maintain mucosal homeostasis (and thus resist tumorigenesis) by management of bacterial populations, either through its potent bactericidal functions (Mulherkar *et al*., 1991; Harwig *et al*., 1995; Weinrauch *et al*., 1998; Qu and Lehrer, 1998; Laine *et al*., 1999) or via the production of lipid signaling mediators, such as arachidonic acid and prostaglandins. A distinct function of prostaglandins in the intestine is to protect against mucosal injury, such as that caused by inflammatory bowel disease (IBD), and promote wound healing (Newberry *et al*., 1999; Cohn *et al*., 1997; Elder *et al*., 1997). One histopathological characteristic of IBD in mice and humans (and also frequently observed in *Min* adenomas, R Cormier, unpublished) is the depletion of the goblet cell population (Fukushima *et al*., 1999). Potential interactions between functions of Pla2g2a (prostaglandin biosynthesis, bactericidal), susceptibility to IBD, and colon tumorigenesis warrant further investigation with appropriate mouse models.

Regional modifiers of neoplasia

In our current study we have reaffirmed the role of *Pla2g2a* in providing intestinal tumor resistance in the

mouse, and we have mapped a second component of *Mom1* that lies distal to *Pla2g2a* and *D4Mit64*. This second locus provides a more modest resistance effect that is restricted to a defined region of the small intestine. Recent studies by our group and others indicate that the mouse intestine consists of discrete subregions that are differentially susceptible to tumorigenesis. Other modifiers of the *Min* phenotype act regionally within the intestine (A Bilger and WF Dove, unpublished results; Cormier and Dove, manuscript submitted), as do several *Apc*-independent tumor regulators such as *TGFβ-1* and *Smad3*, which develop neoplasia that is restricted to the large bowel (Engle *et al*., 1999; Zhu *et al*., 1998). NSAIDs such as piroxicam have been reported to be relatively ineffective in specific regions of the Min intestine, such as the duodenum and colon (Ritland and Gendler, 1999). Thus, it is not surprising that the two components of $Mom1^{AKR}$ exert differential regional effects. In fact, it may turn out to be of greater novelty that *Pla2g2a* demonstrates its resistance phenotype throughout the intestine. One exception to this broad range of resistance, however, is the incidence of duodenal periampullar adenomas. This tumor type occurs in approximately 25% of Min mice, but none of several genetic modifiers recently studied in our laboratory (*Mom1*, $Pla2g2a^{AKR}$, or $Dnmt1^{N/+}$) have affected its development.

Distal Mom1 *candidates*

What is the identity of the second gene in the *Mom1* region? Answering this question will require functional genetic tests such as those that have been performed here for *Pla2g2a*. As with *Pla2g2a*, this approach will initially require suitable test candidates. The *Mom1* locus lies in a region of mouse distal chromosome 4 that is syntenic with human chromosome 1p35-36 (Tischfield *et al*., 1996; Praml *et al*., 1995), a region prone to LOH in a variety of human cancers, including familial and sporadic human colon cancer (Tomlinson *et al*., 1996; Herzog *et al*., 1995; MacPhee *et al*., 1995). The list of candidates from the syntenic region of mouse distal chromosome 4 includes the recently expanding family of type II-$sPla_2$ that have been mapped to the same region of distal chromosome 4 (Valentin *et al*., 1999; Ishizaki *et al*., 1999; Murakami *et al*., 1999). Examples are II-D and II-F, each expressed at low levels in the mouse intestine. We have excluded II-C and V as candidates for the distal resistance gene because we have found that polymorphisms in intron 3 of *Pla2gV* and in the 3′ untranslated region of *Pla2g2c* segregate with the AKR region of recombinant line 25, which lacks the distal AKR resistance allele. In addition, we showed previously that *Pla2gV* lies only 8-kb from *Pla2g2a* (Cormier *et al*., 1997). Another potential candidate in this region is *Hspg2* (heparan sulfate proteoglycan 2), a basement membrane perlecan that has been mapped at 71.4 cM and is polymorphic among inbred strains AKR/J, C3H/HeJ, DBA/2J (*Mom1* resistant and $Pla2g2a^{+}$) and C57BL/6J (*Mom1* sensitive and $Pla2g2a^{-}$). Glypicans, also members of the heparan sulfate proteoglycan family, have been shown to bind and activate Pla2g2a (Murakami *et al*., 1999).

Summary

While not all genetic factors identified in the mouse may prove useful in the genetic epidemiology of the human, or even in treating human colon cancer, the use of inbred mouse strains should continue to identify candidate genes and biochemical pathways for further analysis and potential testing in clinical studies. The work reported here emphasizes both the feasibility of polymorphic modifier identification, such as for *Pla2g2a*, as well as its potential for complexity, as illustrated by our dissection of the *Mom1* region into at least two tightly linked, additive modifiers.

This finding of complexity is not unique. QTL analyses of disease phenotypes in mouse models (of lupus, diabetes, epilepsy and plasmacytoma) have recently identified several clusters of tightly linked modifiers whose individual effects were known only when the modifier region was dissected by fine-structure mapping (Potter *et al*., 1994; Podolin *et al*., 1997, 1998; Legare *et al*., 2000; Morel *et al*., 1999). It remains to be seen to what extent the ascertainment bias generated by modifier clusters complicates the identification of polymorphic modifiers.

Materials and methods

Animal care and breeding

Experimental mice were bred at the McArdle Laboratory for Cancer Research. Mouse husbandry was performed as previously described (Cormier *et al*., 1997).

Strain designations

Strains are designated throughout as B6 (C57BL/6J), B6-Min (B6-$Apc^{Min/+}$, or AKR (AKR/J).

Construction of transgenic lines

Cosmid KH1 was isolated and transgenic line $Pla2g2a^{KH1}$ was generated and maintained as previously described (Cormier *et al*., 1997). Transgenic lines $Pla2g2a^{948}$ and $Pla2g2a^{959}$ were generated by injecting a purified 41-kb insert from cosmid KH1 into pronuclei of fertilized B6 mouse eggs. This procedure was conducted by the UW-Madison Biotechnology Center Transgenic Animal Facility. Transgenic mice were screened by PCR with DNA prepared from tail biopsies as described (Cormier *et al*., 1997).

Experimental classes of mice

The B6-Min pedigree has been maintained by backcrossing B6-$Apc^{Min/+}$ males to B6 females ($n>45$). The B6-$Mom1^{AKR}$ congenic strain has been maintained as previously described (Gould *et al*., 1996a) ($n\geqslant 14$). Crosses involving $Pla2g2a^{AKR}$ transgenic line KH1 have been previously described (Cormier *et al*., 1997). Females from $Pla2g2a^{AKR}$ transgenic lines 948 and 959 were crossed to B6-Min males to produce Min progeny carrying the transgene. Non-transgenic Min siblings were used as controls. Recombinant lines 29, 57, 63, and 52 were generated and have been maintained as previously described (Gould *et al*., 1996b; $n=10-12$ backcross generations). Females from recombinant lines 29, 52, 57, and 63 were crossed to B6-Min males. Min progeny carrying recombinant chromosomes were compared with Min sibling control animals that were homozygous for B6 alleles. All mice were sacrificed at 90 days of age unless otherwise

indicated. Age-matched Min $Mom1^{AKR/B6}$ congenic mice were also used for comparison in this analysis.

Intestinal tumor scoring and sizing

All mice were sacrificed by CO_2 asphyxiation. The entire intestinal tract was removed, prepared, and fixed as previously described (Cormier *et al*., 1997). Tumors ($\geqslant$0.4 mm for the $Pla2g2a^{AKR}$ transgenic experiments and $\geqslant$0.15 mm for the recombinant studies) were scored from fixed tissues with a Nikon SMZ-U dissecting microscope at 10× (transgenic studies) or 20× (recombinant studies) magnification. Tumor sizes were estimated by measuring the maximum diameter of tumors from the small or large intestine with a calibrated eyepiece reticle. In each experiment, all tumors were counted and sized by a single observer who was blind to the genotype of the samples.

Genotyping

DNA was isolated from blood as described previously (Gould *et al*., 1996b). The genotype of the *Apc* locus was determined by a PCR assay as described (Dietrich *et al*., 1992). The *Mom1* genotype was assigned on the basis of PCR analysis of the closely flanking markers *D4Mit54* and *D4Mit13* (for the transgenic experiment) and *D4Mit12*, *D4Mit13*, and *D4Mit180* (recombinant experiment) as described (Gould *et al*., 1996a). The *Pla2g2a* transgenic genotype was determined by PCR using the primers T7 and Pla47b as previously described (Cormier *et al*., 1997). In the recombinant experiments, *Pla2g2a* genotype was determined by PCR using the primers Pla2s8F and Pla2s144R (Gould *et al*., 1996a). Mice carrying recombinant chromosomes (lines 29, 57, 63) were designated recombinant if they were heterozygous for flanking markers as described (Gould *et al*., 1996b; also describes chromosome 4 markers used in this analysis).

Western-blot analysis

The intestine was removed, opened, washed in phosphate-buffered saline (PBS), divided into segments, and minced with a razor blade. The tissue was suspended in 1 M acetic acid and incubated overnight at 4°C. Samples were centrifuged at 4000 r.p.m. in a swinging bucket rotor for 30 min, and the supernatant was transferred to a clean tube and stored at −20°C. Western blot analysis was performed as described (Halberg and Kroos, 1992), except that 0.1 volume of 10 N NaOH was added to samples before electrophoresis and blots were probed with a 1 : 2000 dilution of rabbit polyclonal antiserum against Pla2g2a (Mulherkar *et al*., 1993). Immunodetection with goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad) and ECL Western blotting kit (Amersham) was performed according to the manufacturer's instructions. All signals were within the linear response range, as determined by a titration curve that was generated by loading increasing amounts of extract prepared from the small intestine of transgenic animals. Signals were quantitated with a Molecular Dynamics densitometer and software.

Immunohistochemistry

The Pla2g2a antiserum (Mulherkar *et al*., 1993) was used, at a 1 : 30 000 dilution, for standard peroxidase-based immunohistochemistry. The intestine was cut longitudinally, rinsed in 1× PBS, washed, transferred to 70% ethanol, embedded in paraffin, and serially sectioned (5 μM). The sections were then dewaxed and rehydrated through a graded ethanol series. Antigen retrieval was performed by heating the slides in citrate buffer (pH 6) for 25 min on full power in a 650-W Kenmore microwave oven. Endogenous peroxidase activity was quenched by incubation of the slides in 0.03% hydrogen peroxide solution for 15 min at room temperature. Immunohistochemistry was performed with the ABC elite peroxidase system (Vector) according to the manufacturer's instructions. Sections were developed in diaminobenzidine substrate (Sigma) and counterstained with hematoxylin for 5 s.

Alcian blue staining

To establish the identity of cells in the large intestine expressing Pla2g2a, adjacent serial sections were stained with alcian blue (8GX), pH 2.5. This stain detects the acidic mucopolysaccharides expressed by goblet cells. Sections were deparaffinized and rehydrated in distilled water, incubated in 3% acetic acid solution for 3 min, stained in alcian blue solution for 30 min, washed in running water for 10 min, rinsed in distilled water, counterstained in filtered nuclear fast red (Kernechtrot) solution for 5 min, washed in running water for 1 min, and dehydrated through an ethanol series.

Statistics

One-sided *P* values for intestinal tumor numbers and sizes were determined by comparison of each test class with contemporaneous B6-Min control mice (generated from the same cross) by use of the non-parametric Wilcoxon Rank Sum test. These tests were performed with the MStat statistical analysis software (N Drinkwater, UW-Madison, WI, USA).

Abbreviations

APC/*Apc*, adenomatous polyposis coli gene in humans/mice; Min/*Min*, *M*ultiple *i*ntestinal *n*eoplasia [note: *Min* is also used in place of $Apc^{Min/+}$ and Min is used to indicate mice and tumors]; B6, C57BL/6J; *Mom1*, *M*odifier *o*f *M*in-1; LOH, loss-of-heterozygosity; AKR, AKR/J

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