BASIC–ALIMENTARY TRACT

Linkage to Peroxisome Proliferator-Activated Receptor-γ in SAMP1/YitFc Mice and in Human Crohn's Disease

KAZUHIKO SUGAWARA,[#] TIMOTHY S. OLSON,[†] CHRISTOPHER A. MOSKALUK,[§] BRIAN K. STEVENS,[‖] SHARON HOANG,* KOSUKE KOZAIWA,** FABIO COMINELLI,* KLAUS F. LEY,[†] and MARCIA MCDUFFIE*,[‖]

*Departments of Internal Medicine, [†]Physiology and Biological Physics [§]Pathology, [‖]Microbiology, and [¶]Biomedical Engineering, University of Virginia Health System, Charlottesville, Virginia; [#]Sendai City Hospital, Sendai, and **Nippon Boehringer-Ingelheim, Medical Division, Hyogo, Japan

See editorial on page 501.

Background & Aims: **Genetic predisposition is implicated strongly in Crohn's disease. Disease-associated mutations in *NOD2/CARD15*, the best-studied susceptibility gene in this disorder, explain only a small fraction of the heritability. The SAMP1/YitFc (SAMP1/Fc) mouse strain expresses many features of Crohn's disease in humans. We bred SAMP1/Fc to disease-resistant AKR mice to identify additional susceptibility genes that may play a role in human disease.** ***Methods:*** **Linkage disequilibrium mapping was performed in an (AKR × SAMP1/Fc) backcross to SAMP1/Fc, followed by sequencing, expression analysis using reverse transcription polymerase chain reaction (PCR) and immunohistochemistry, and functional testing in vivo of the regional candidate gene encoding the peroxisome proliferator-activated receptor γ (*Pparg*). A cohort-based association study was performed in humans.** ***Results:*** **We show that ileitis is blocked in SAMP1/Fc mice by inheritance of AKR alleles on chromosome 6 in the region of *Pparg*. Major differences in Pparγ expression in the parental mouse strains are found specifically in the crypts of the small intestine, and treatment of ileitis-prone mice with a Pparγ agonist decreased disease severity in susceptible mice expressing low levels of the protein. Rare alleles of *PPARG* are associated significantly with Crohn's disease in humans.** ***Conclusions:*** **We have identified *Pparg* as a susceptibility gene in both the SAMP/Yit mouse and in human Crohn's disease. Similarities between Crohn's disease and the SAMP1/Fc model suggest that the effect of this gene in humans may be mediated through regulation of PPARγ activity in the crypts of the small intestine.**

SAMP1/YitFc (SAMP1/Fc) mice spontaneously develop chronic inflammation of the ileum with many characteristics of classic Crohn's disease (CD) in humans, including discontinuous and transmural inflammatory lesions occurring mainly in the terminal ileum.[1–3] Although histology is unremarkable in these mice at 4 weeks of age, ileitis is present in nearly 100% by 10 weeks.[4] The lesions contain mixed infiltrates of neutrophils and mononuclear cells, including lymphocytes. Villus atrophy and crypt hyperplasia, characterized by crypt elongation with increased mitotic figures and increased numbers of Paneth cells, are localized to inflamed regions in mice with established disease. In contrast to most other mouse models of inflammatory bowel disease,[5] SAMP1/Fc mice develop no significant inflammation in the colon and the inflammation of the ileum persists as the animals age. Associated findings include inflammatory skin lesions and perianal fistulizing disease in a minority of the animals,[4] as well as nondestructive liver infiltrates in the majority after 30 weeks.[1]

We previously showed that SAMP1/Fc is a recombinant inbred strain derived from AKR and an unknown donor unrelated to most of the commonly used inbred mouse strains.[3] In crosses with nonautoimmune C57BL/6J mice, we previously identified a susceptibility locus on chromosome 9, *Ibdq1*, which appears to regulate inflammation-associated epithelial cell changes in an additive fashion.[3] This locus and a second suggestive locus on chromosome X map to regions of the genome carrying predominantly AKR alleles. After stratifying by genotype at the locus on chromosome 9, sequential regression analyses of a (C57BL6/J × SAMP1/Fc)F_2 cross revealed a second suggestive linkage on chromosome 6 in a region largely derived from the non-

Abbreviations used in this paper: NF-κB, nuclear factor κ B; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; QTL, quantitative trait locus; SAMP1/Fc, SAMP1/YitFc; SNP, single-nucleotide polymorphism.

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AKR parental strain.[3] AKR mice do not develop intestinal inflammation, and we postulated that the locus on chromosome 6 might represent a required non-AKR contribution to the phenotype of ileitis in SAMP1/Fc mice. This hypothesis was tested using a backcross of AKR mice to SAMP1/Fc, and candidate susceptibility genes were identified through mapping studies in this population.

Materials and Methods

Animals

SAMP1/Yit mice[1] have been propagated as a specific pathogen-free colony for more than 20 generations under protocols approved by the Institutional Animal Care and Use Committee following American Association of Accreditation of Laboratory Animal Care guidelines. We now distinguish this line from the original strain as SAMP1/YitFc.[4] AKR/J (AKR) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Female AKR and (AKR × SAMP1/Fc)F_1 mice bred to male SAMP1/Fc mice produced F_1 and backcross mice, respectively. For treatment studies, ileitis-prone (C57BL/6J × SAMP1/Fc)F_1 mice were generated using C57BL/6J (B6) mice bred in our colony from stock obtained from The Jackson Laboratory.

Histology

The terminal 15 cm of the small intestine (ileum), stained with H&E, was scored by an experienced intestinal pathologist (C.A.M.), who was blind to the experimental design. Both the severity and extent of lesions were determined by using indices for active inflammation (granulocytic), chronic inflammation (monocytic), and epithelial changes as previously described.[3] A calculated total inflammatory score is the sum of the 3 indices.

Peroxisome proliferator-activated receptor γ (Pparγ) was stained in Bouin's-fixed tissues using a rabbit polyclonal IgG (Santa Cruz Biochemicals, Santa Cruz, CA) after permeabilization with .1% saponin, followed by a biotinylated anti-rabbit IgG and avidin-biotin complex (Vector Laboratories, Burlingame, CA). Controls, prepared by replacing the primary Pparγ antiserum with 3% bovine serum albumin, were uniformly negative.

Genetic Analysis

For quantitative trait locus (QTL) analysis, mice were genotyped by polymerase chain reaction (PCR) using fluorochrome-labeled oligonucleotides specific for a panel of informative microsatellite loci[3] as previously described.

Informative polymorphisms in *Ogg1*,[6] *Tnfrsf1a*,[7] and *Prh1*[8] were identified by PCR using published oligonucleotide sequences. Additional polymorphisms among the SAMP1/Fc, AKR, and B6 strains, as well as human *PPARG*-associated polymorphisms, were identified through sequencing of selected genes by standard methods. Genbank accession numbers for new sequences are as follows: *Bid*: AY390522–AY389524; *Ogg1*: AY389525–AY389527; and *Pparg*: AY389528–AY389545. Primers, selected from the relevant public databases of genomic sequences, were synthesized commercially (MWG-Biotek, High Point, NC); sequences are available on request.

Candidate genes were identified based on evidence of expression in cells of the peripheral immune system or in intestinal epithelium using publicly available expression databases (http://www.informatics.jax.org/searches/expressionmenu.shtml[9]; and http://expression.gnf.org/cgi-bin/index.cgi[10]), as well as standard literature search engines. Transcription factor binding sites were identified using TFSearch (http://www.cbrc.jp/research/db/TFSEARCH.html), an algorithm based on TFMatrix.[11]

Quantitative PCR

Jejunum and ileum were sampled using a 1-cm segment of small intestine 2–3 cm distal to the emergence of the duodenum from the retroperitoneum and 1 cm immediately proximal to the cecum, respectively. RNA from all tissues was extracted using Qiashredder columns and an RNAeasy isolation kit (Qiagen, Valencia, CA). Real-time reverse-transcriptase PCR for *Pparg1* was performed in triplicate for each sample using a forward primer within exon A1 (5′- GGG CTGAGGAGAAGTCAC-3′) and a reverse primer spanning the intersecting regions of exons A1 and A2 (5′-TTCTT-TCAAATCTTGTCTGTCAC-3′) with SYBR Green labeling (Quantitect; Qiagen) in an iCycler (BioRad, Hercules, CA). Values were normalized to a single B6 liver sample run as 3.3-fold dilutions of the template RNA.

Isolation of Intestinal Epithelial Cells

Using a modification of published methods,[12] the terminal 15 cm of small intestine was incubated for 10 minutes with shaking in HEPES-buffered Hanks' balanced salt solution containing 3 mmol/L ethylenediaminetetraacetic acid, .05 mmol/L dithiothreitol, and 100 mmol/L N-acetylcysteine, decanted through a 70-μm mesh filter and washed repeatedly before resuspension of cells in RPMI with 10% fetal bovine serum. Epithelial cells were separated from intraepithelial lymphocytes using a Percoll gradient (Amersham Biosciences, Piscataway, NJ) and flash-frozen in liquid nitrogen. RNA was extracted as described previously.

Drug Treatment

Thirty-week-old (B6 × SAMP1/Fc)F_1 mice were treated orally for 4 weeks with either 10 or 60 mg/kg per day rosiglitazone (Calbiochem, San Diego, CA). In experiment 1, rosiglitazone was dissolved at 10 mg/mL in 100% ethanol and diluted with autoclaved water supplemented with ethanol to match the intake in the high-dose rosiglitazone treatment group. Water intake, monitored daily, remained stable for all groups. Intraperitoneal treatment with 100 μg/day dexamethasone for 3 days before termination was used as a positive control for therapeutic effect. After experiment 1, rosiglitazone was no longer available from the same source. In subsequent experiments, SAMP1/Fc or (B6 × SAMP1/Fc)F_1 mice were fed either

Table 1. Cohort Characteristics

Characteristics	CD	Control
Female	56.0%	74.4%
Ethnicity		
Caucasian	94.0%	93.6%
African American	4.5%	3.2%
Other	1.5%	3.2%
Age at diagnosis	29 ± 14.1	–
<21 years at diagnosis	31.3%	–
Disease location[a]		
Ileum only	32.3%	–
Ileum and colon	42.7%	–
Colon only	25.0%	–
Perianal disease	15.7%[b]	–

[a]Seven individuals were without a recorded site of disease.
[b]Includes 2 individuals with documented disease restricted to perianal area.

measured amounts of normal pulverized chow or pulverized chow containing .4 g/kg rosiglitazone (GlaxoSmithKline, Essex, UK) for 4 weeks. Body weights, measured every 3 days, remained stable throughout the experiment in all groups.

Genetic Association Study

Peripheral blood DNA samples from 134 unrelated individuals with clinically confirmed CD were obtained from the inflammatory bowel disease tissue collection at the University of Virginia. Disease characteristics and ethnic distribution are representative of the population seen at this tertiary care center (Table 1). The collection also provided an ethnically matched set of 125 DNA samples from local healthy volunteers. The use of samples from this collection for genetic analysis without identifying information was approved by the local Human Investigations Committee. Genomic DNA was isolated and genotyped using PCR/restriction fragment length polymorphism methods for 3 single-nucleotide polymorphisms spanning the coding region for *PPARG1*: single-nucleotide polymorphism 1 (SNP1) = G12350898A (rs2067819); SNP2 = G12359887A (rs3892175); and SNP3 = C12467406T (rs3856806). Primer sequences and restriction enzymes are available on request.

Statistical Analysis

QTL analysis was performed using Map Manager QTb29ppc and the results are expressed as the likelihood ratio statistic that approximates a χ^2 value.[13] Levels of significance were determined using the permutation function with 5000 replicates. Threshold values of the permutation test (suggestive, significant, and highly significant) correspond to genome-wide P values of .36, .05, and .01, respectively. For the treatment study, comparisons of experimental groups were performed using the Mann–Whitney U test. Association studies for *PPARG* were performed using a 3 × 2 contingency table for χ^2 analysis.

Results

Histologic Analysis Suggests a Dominant Protective Effect at a Single Locus by AKR Alleles

Ileitis of variable severity is found in nearly all SAMP1/Fc mice after 10 weeks of age (total inflammatory score: median, 11.25; range, 1.5–16.5; $n = 16$), and the lesions persist throughout the remaining life of the mice.[1–4] In contrast, AKR mice display little evidence of inflammation (range of total inflammatory scores, .25–1.5 by 30 weeks of age; $n = 4$), even after sharing cages with SAMP1/Fc mice for >3 months before examination. Similarly, we found little evidence of inflammation among (AKR × SAMP1/Fc)F_1 mice housed with their SAMP1/Fc fathers until 28 days of age (total inflammatory scores: median, .0; range, 0–2.0; and median, .5; range, 0–4.0, at 10 and 30 weeks, respectively; $n = 10$ and 12). This shows that ileitis is functionally recessive in the (AKR × SAMP1/Fc) cross and is unlikely to be inducible by intestinal flora in the absence of a fully susceptible genetic background.

To estimate the number of protective loci in AKR, we backcrossed (AKR × SAMP1/Fc)F_1 mice to SAMP1/Fc ($n = 89$). By 10 weeks of age, nearly half of the back-cross mice showed moderate to severe inflammatory changes sim-

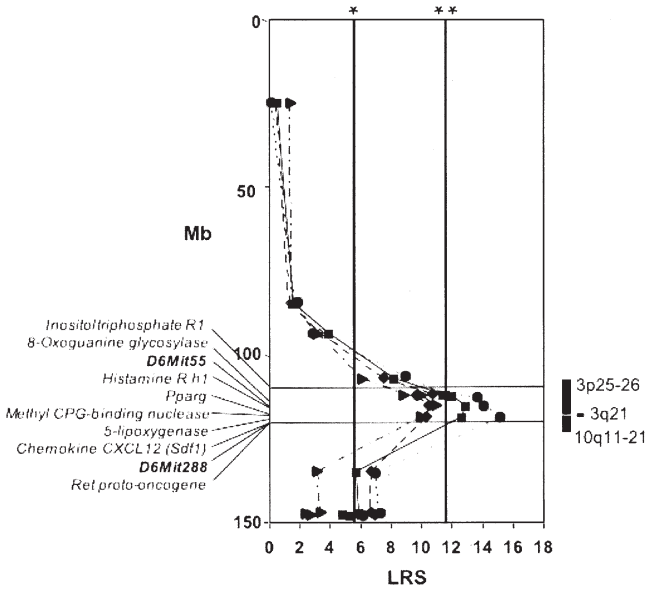

Figure 1. QTL analysis links susceptibility to chromosome 6. Individual inflammatory indices and the composite total inflammatory score for the (AKR × SAMP1/Fc) backcross cohort (n = 89) were tested for evidence of linkage by a single-point quantitative trait analysis. Levels of significance at the genome-wide level are shown by vertical bars: *P < .37 (suggestive); **P < .05 (significant). ◆, Active index; ●, chronic index ▲, epithelial index; ■, total inflammatory score. Regions of conserved synteny with the human genome are shown at the right (*filled bars*), along with relative locations of selected regional candidate genes (*left*).

Table 2. QTL Analyses of (AKR × SAMP1/Fc) Backcross and (B6 × SAMP1/Fc)F_2 Mice Show Colocalization

Locus	Megabase (10^6 *bases*)	Total score		Active index		Chronic index		Epithelial index	
		LRS	*P*	LRS	*P*	LRS	*P*	LRS	*P*
(AKR × SAMP1/Fc) backcross: *Ibdq2*									
D6Mit8	84.6	1.6	.19	1.3	.26	1.7	.19	1.5	.22
D6Mit31	93.5	3.9	.047	3.3	.068	3.0	.082	3.1	.078
D6Mit149	106.8	8.2	.0043	7.5	.0060	9.2	.0025	6.1	.014
D6Mit104	111.8	11.4	.0008	10.7	.0011	11.6	.0007	8.8	.0030
D6Mit108	112.1	12.0	.0005	9.7	.0019	13.9	.0002	10.0	.0016
Ogg1	114.2	12.9	.0003	10.5	.0012	14.0	.0002	11.0	.0009
D6Mit55	115.1	12.9	.0003	10.5	.0012	14.0	.0002	11.0	.0009
Pparg	116.2	12.9	.0003	10.5	.0012	14.0	.0002	11.0	.0009
D6Mit288	118.2	12.6	.0004	10.3	.0013	15.3	.0001	10.0	.0016
Bid	121.7	8.3	.0039	8.3	.0039	9.5	.0021	5.8	.016
Tnfrsfla	126.2	7.7	.0055	7.9	.0048	8.3	.0041	5.4	.020
Prh1	132.9	6.0	.014	6.4	.011	6.5	.011	4.0	.045
D6Mit14	134.7	5.7	.017	6.7	.0097	7.1	.0076	3.1	.077
D6Mit15	147.1	5.9	.015	6.7	.0099	7.2	.0073	3.4	.064
D6Mit201	147.3	4.8	.028	6.1	.013	6.0	.014	2.4	.12
D6Mit373	147.8	5.3	.022	6.9	.084	6.1	.014	2.7	.10
(B6 × SAMP1/Fc)F_2[a]									
D6Mit16	72.2	10.7	.0048	9.4	.0093	5.8	.055	10.4	.0056
D6Mi243	73.2	10.9	.0042	9.3	.0096	6.3	.043	10.5	.0053
D6Mi39	106.8	9.6	.0083	6.1	.048	5.7	.057	11.7	.0029
Ogg1	114.2	9.8	.0073	7.9	.019	5.1	.078	10.2	.0061
D6Mi55	115.2	8.6	.014	6.5	.040	4.8	.090	8.9	.012
Pparg	116.2	6.6	.037	4.7	.097	4.0	.14	6.9	.031

LRS, likelihood ratio statistic.
[a]LRS values after stratification by genotypes at loci on chromosome 9 near *Ibdq1* using the multiple regression function of Map Manager QT.

ilar to those seen in age-matched SAMP1/Fc mice (44.9% vs. 87.5% with total inflammatory scores >5.0 for backcross vs. SAMP1/Fc mice, respectively), suggesting the effects of a single locus.

QTL Analysis Identifies a Single Susceptibility Locus on Chromosome 6

SAMP1/Fc and AKR mice carry distinguishable microsatellite alleles at slightly more than 50% of 243 loci tested in our original analysis.[3] AKR-derived intervals comprise more than half of chromosomes 2, 4, and 10, and we found only 1 or 2 non-AKR microsatellites on chromosomes 7, 9, 17–19, and X. However, a selected panel of 43 polymorphic microsatellites allowed us to screen each chromosome in the backcross cohort and perform a QTL analysis based on the severity of ileitis. We found significant evidence for linkage only on chromosome 6, with a peak likelihood ratio statistic of 15.3 ($P = .0001$) at *D6Mit288* (Figure 1). We have designated this locus *Ibdq2* (inflammatory bowel disease-QTL2). There was no suggestion of primary linkage to any other chromosome. The *Ibdq2* interval overlaps the suggestive QTL previously identified in (C57BL/6 × SAMP1/Fc)F_2 mice detected only after controlling for genotypes at a primary locus on chromosome 9 (Table 2).[3] Because we had shown previously that *Ibdq1* maps to a region carrying functionally dominant susceptibility alleles probably of AKR derivation, it was expected that this locus would not be detected in the AKR backcross.

Genes of known function encoded between *D6Mit149* and *D6Mit14*, along with their human homologs, were identified using Build 30 of the Mouse Genome Sequencing Consortium data via the Ensembl Genome Browser (Table 3).[14] Polymorphic microsatellite loci near *Tnfrsf1a* and *Prh1*, as well as selective sequencing of *Bid*, *Ogg1*, and *Pparg*, confirmed that the SAMP1/Fc alleles of these genes are unrelated to those in AKR, and also narrowed the candidate region to the *D6Mit149-Bid* interval (Table 2). Within this interval, the genes known to be expressed in either the peripheral immune system or the intestinal epithelium in mice map to 2 distinct human chromosomes (Table 3; Figure 2). These include the 3(p21–p26) region previously suggested to encode a susceptibility locus for human CD in 4 independent genome-wide scans (reviewed in [15]).

Pparg Fulfills Criteria for a Susceptibility Gene in SAMP1/Fc Mice

The *Pparg* gene is an obvious candidate for *Ibdq2* because of its role in suppressing inflammatory responses.[16] In mice, 2 isoforms, Pparγ1 and Pparγ2, are regulated differentially and show small differences in N-terminal

Table 3. Candidate Genes for *Ibdq2*

Mb[a]	Symbol	Descriptive name	Human mapping
100.4	*Prok2*	Prokineticin-2	3p13
107.4	*Il5ra*	IL-5 receptor α	3p26.2
109.0	*Itpr1*	Inositol triphosphate receptor 1	3p26.1
114.1	*Ogg1*	Oxoguanine DNA glycosylase	3p25.3
115.3	*Hrh1*	Histamine receptor h1	3p25.3
116.1	*Pparg*	Peroxisome proliferator–activated receptor γ	3p25.2
116.5	*Mdb4*	Methyl CPG-binding endonuclease	3q21.3
117.1	*Alox5*	Arachidonate 5-lipoxygenase	10q21.1
117.8	*Sdf1*	Stromal cell–derived factor 1 (CXCL12)	10q11.21
118.8	*Ret*	c-Ret proto-oncogene	10q11.21
120.1	*Wnt5b*	Wingless homolog, 5b	12p13.33
121.1	*Il17r*	IL-17 receptor	22q11.22–23
121.5	*Atp6v1e1*	Atpase, proton pump regulatory subunit	22q11.21
121.6	*Bid*	BH3-interacting death domain agonist	22q11.21
123.2	*Klrg1*	Killer cell lectin-like receptor γ1	12p13.31
123.5	*Apobec1*	apoB messengerRNA editing enzyme	12p13.31
125.5	*Bcap37*	B-cell–associated inhibitory receptor	12p13.31
125.6	*Eno2*	Enolase	12p13.31
125.7	*Cd4*	CD4	12p13.31
126.0	*Tnfrsf7*	CD27	12p13.31
126.1	*Ltbr*	Lymphotoxin β receptor	12p13.31
126.2	*Tnfrsf1a*	Tumor necrosis factor receptor 1	12p13.31
126.3	*Cd9*	CD9	12p13.31
126.9	*Nf3*	Neurotrophin 3	12p13.31
127.9	*Ccnd2*	Cyclin D2	12p13.32
130.1	*Cd69*	CD69	12p13.31
130.4	*Klrd1*	Killer cell lectin-like receptor δ1	12p13.2
132.8	*Prh1*	Proline-rich protein, Hae III subfamily 1	12p13.2
135.2	*Cdkn1b*	Cyclin-dependent kinase inhibitor, 1b	12p13.2
135.7	*Emp1*	Epithelial membrane protein 1	12p13.1
136.0	*Grin2b*	Glutamate receptor, inotropic (NMDA)	12p13.1

IL, interleukin.
[a]Mb from the origin of chromosome 6.

sequence resulting from the use of alternative exons.[17] Regulation of expression of these isoforms is similar in humans, with PPARγ1 found in most tissues at varying levels and PPARγ2 expression limited to liver and fat.[18] Multiple polymorphisms distinguish AKR from SAMP1/Fc upstream of the transcription start site for Pparγ1 and in the A1 and A2 exons encoding most of its 5′ untranslated region. In particular, a 7-bp deletion in the AKR *Pparg1* promoter alters spacing between linked consensus binding sites for the homeobox transcription factors Cdx1 and Nkx-2 (Figure 2*A*). A similar Cdx1/Nkx2 cluster also is found in the human *PPARG1* promoter (Figure 2*A*), despite the complete lack of sequence homology between human and mouse A1 and A2 and the 2000 bases upstream of A1.[17] Cdx1 is critical for fetal gut development[19] and is expressed selectively in the crypts of small intestine and the colon of adults.[20] Nkx-2.3 also is expressed selectively in the crypts of the small intestine, as well as in the spleen, and deletion of *Nkx-2.3* leads to significant defects in the architecture of both the spleen and small intestine.[21] The demonstration that a similar configuration of 3 possible Cdx1 sites adjacent to a Nkx2 consensus site is preserved in the human *PPARG1* promoter, despite the complete lack of sequence homology between human and mouse A1 and A2 and the 2000 bases upstream of A1,[17] suggest that Pparγ1 expression in the crypts of the small intestine is critical for normal development and/or function of this tissue.

Expression and Localization of Pparγ in AKR and SAMP1/Fc Mice

Differences between SAMP1/Fc and AKR mice in the *Pparg1* proximal promoter suggested possible differential expression of this isoform, particularly in the small intestine where Cdx1 and Nkx-2.3 are important transcriptional regulators.[20,21] Quantitative PCR using *Pparg1*-specific primers showed low levels of expression in splenic and mesenteric lymph node leukocytes that did not differ significantly by strain (Figure 2*B*). However, liver-specific messenger RNA levels were lower in AKR mice than in either SAMP1/Fc or B6 mice, whereas levels in both ileum and jejunum were higher in AKR mice and B6 mice than in SAMP1/Fc. These differences strongly suggest that tis-

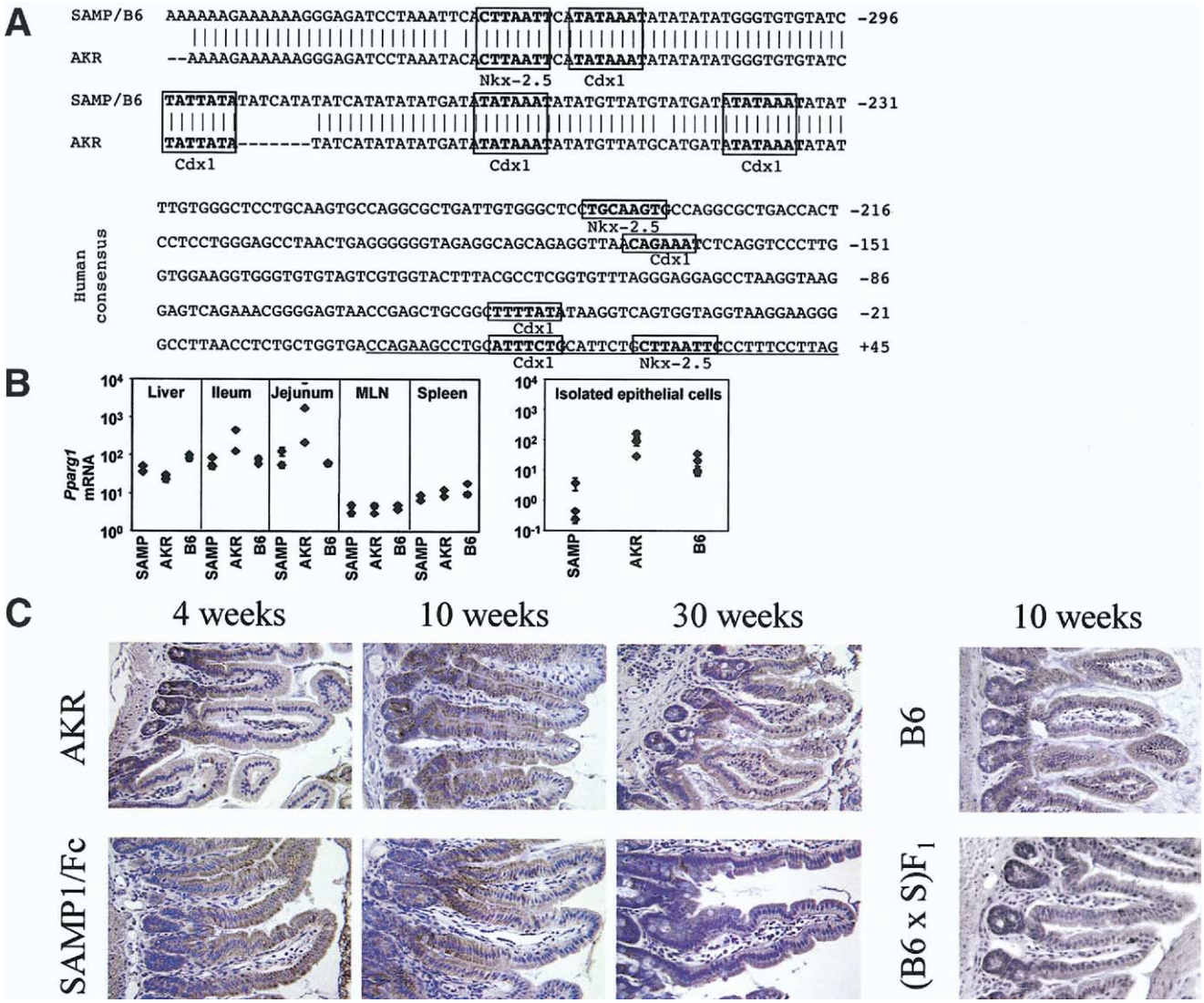

Figure 2. Differences in Cdx1 consensus binding sites are associated with altered expression of *Pparg1*. (*A*) Sequence analysis of the *Pparg1* promoter revealed major differences in spacing among a cluster of Cdx1 consensus binding sites. A putative Nkx-2 binding site in close proximity to the Cdx1 cluster also is shown. A conserved cluster of Cdx1 and Nkx-2 consensus binding sites also was found in the human gene (exon 1, *underlined*). (*B*) *Left panel*, quantitative allele-specific PCR was performed on messenger RNA isolated after careful dissection from associated fat. Data are representative of 2 separate experiments on 2 individual mice from each strain. *Right panel*, comparable differences also were seen in messenger RNA levels from isolated small intestine epithelial cells from each mouse strain (SAMP1/Fc, N = 3; B6 and AKR, N = 4). Error bars representing 1 SD are shown. Note $\log_{10}$ scale. (*C*) Representative sections displaying comparable villus architecture (from 3 individual mice in each strain at relevant ages) shows dramatic differences in localization and intensity of Pparγ staining.

sue-specific regulation of *Pparg1* expression is extremely complex. We confirmed the strain-specific differences between AKR and SAMP1/Fc in the small intestine by using isolated epithelial cells (range of relative *Pparg1* levels, 25.2%–222.0% vs. .3%–5.6% of B6 liver standard, respectively). Expression in B6 epithelial cells was intermediate, once again suggesting complexity in the regulation of expression of this gene (Figure 2*B*).

We also found marked differences in localization of Pparγ protein in the small intestine by immunohistochemistry. Intense Pparγ staining typically was seen in the crypt and lower third of each villus in AKR mice at 4 weeks of age, whereas the weaker immunoreactivity in SAMP1/Fc small intestine consistently was absent from the crypt, extending only from the base to the tip of villi (Figure 2*C*). Although some minor distortion of villus architecture is seen in SAMP1/Fc mice at 4 weeks, there is no detectable chronic or acute inflammation at this age. Differences in the localization of Pparγ along the villi were less marked in mice tested at 10 or 30 weeks of age, with expression in all mice extending toward the tips of some villi at the later time points (Figure 2*C*). However, Pparγ staining consistently was absent from the crypts of SAMP1/Fc mice, whereas crypt expression

persisted in AKR mice at all ages tested. As expected, crypts stained positively for Pparg in both (B6 × SAMP1/Fc)F_1 and B6 mice at 10 weeks, although staining intensity appeared to be less than in AKR mice (Figure 2*C*). Pparγ expression in colon was relatively weak compared with small intestine and was limited to epithelial cells adjacent to the gut lumen (data not shown). The pattern of localization we found in colonic epithelium is consistent with the report of Lefebvre et al[22]; however, the intensity of staining in colonic epithelium appeared to be less in all 3 strains than that in crypts from the small intestine of AKR mice, making it difficult to assess relative expression levels among the 3 strains (data not shown). Antibody binding primarily was cytoplasmic in all tissues examined.

Activation of Pparγ Activity Decreases Intestinal Inflammation in (B6 × SAMP1/Fc)F_1 Mice, But Not in SAMP1/Fc Mice

Activation of Pparγ by its natural ligands (unknown) or by synthetic agonists is required for nuclear translocation and transcriptional activity.[16] To test whether enhanced activity of Pparγ could reverse disease in the SAMP1/Fc model, we treated 30-week-old (B6 × SAMP1/Fc)F_1 mice with established ileitis using the synthetic Pparγ agonist rosiglitazone. We previously showed that (B6 × SAMP1/Fc)F_1 mice, unlike (AKR × SAMP1/Fc)F_1, develop ileitis of moderate severity by this age.[3] In a pilot experiment, high-dose oral rosiglitazone (60 mg/kg per day) decreased the total inflammatory score to a level equivalent to a 3-day pulse of dexamethasone (decreases of 56% vs. 55% for rosiglitazone and dexamethasone, respectively; $P \leq .05$; $n = 3–4$ per group). Low-dose rosiglitazone treatment (10 mg/kg per day) decreased the score by 25%, but the difference from control scores was not statistically significant ($P = .14$). In 2 larger experiments, high-dose rosiglitazone treatment again decreased the total inflammatory score by 50%, confirming the preliminary result in the smaller groups of mice ($P = .006$; $n = 11–13$ per group).

In striking contrast to the results in (B6 × SAMP1/Fc)F_1 mice, we found no significant difference in the severity of ileitis after identical treatment of inbred SAMP1/Fc mice. Indeed, there was a tendency to increased severity in the group treated with high-dose rosiglitazone (total inflammatory scores, 8.9 vs. 12.8 for vehicle- and rosiglitazone-treated groups; $n = 11$ and 12 per group, respectively; $P = .10$), suggesting that expression specifically in the crypts of the small intestine was required for the protective effect of enhanced Pparγ activation.

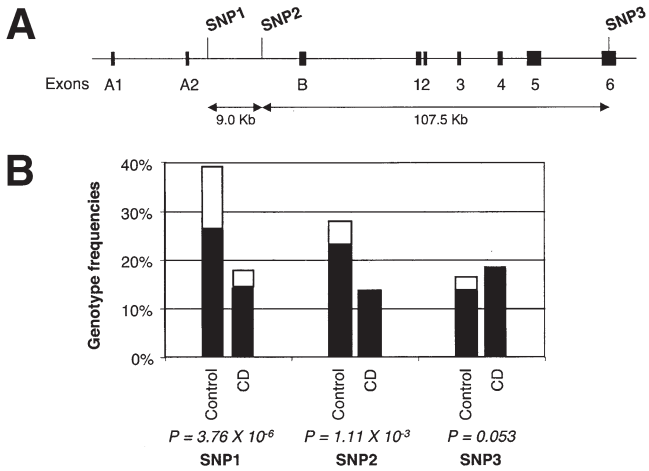

Figure 3. *PPARG* is associated with CD in human populations. (*A*) A diagram of the *PPARG* locus shows the relationship between coding (exons 1–6) and the 5′ untranslated regions of *PPARG1* (exons A1, A2) and *PPARG2* (exon B), with locations of SNP1–3. (*B*) Genotype frequencies for individuals carrying the rare variant for SNP1–3: ■, heterozygotes; □, homozygotes. Genotype frequencies did not differ significantly from those expected based on a Hardy–Weinberg distribution ($P > .20$).

PPARG Is Associated With CD in Humans

Our data suggested a direct genetic relationship between *Pparg* and Crohn's-like ileitis in SAMP1/Fc mice. To test the relationship between *PPARG* alleles and CD in humans, we identified 3 SNPs in *PPARG*, for which the minor variant was found in at least 10% of chromosomes from a sample of 125 unaffected individuals. SNP1 and SNP2 are located in the intron between exon A2 and coding exon 1 (Figure 3*A*). Allele frequencies in a cohort of 134 individuals with well-characterized CD differed significantly from those in an ethnically matched control population ($P < 10^{-5}$ for SNP1; Figure 3*B*). For both SNP1 and SNP2, the rare variant was more common in controls than affected individuals. The bulk of this effect was contributed by differences from control allele distributions among the individuals with colonic involvement, with or without evidence of ileitis. However, because only one third of the cohort had disease restricted to the ileum, the numbers are too small to draw firm conclusions about the relationship between *PPARG* alleles and disease localization. A third distant polymorphism (SNP3), encoding a silent base change in coding exon 6 of the gene (C1431T: H449H), showed only weak evidence for association with CD ($P > .05$).

Discussion

We have shown that allelic differences in *Pparg* can regulate expression of an underlying genetic susceptibility to Crohn's-like ileitis in mice. We also have

shown that minor allelic variants of *PPARG* are associated strongly with disease resistance in humans. Other groups have proposed that PPARγ plays a role in susceptibility to inflammatory bowel disease in humans (reviewed in Daynes and Jones[16]), and we now show positive evidence for association with Crohn's disease. *PPARG* maps to 3(p25–p26) in humans. Four independent studies suggest that at least one susceptibility locus for inflammatory bowel disease also maps to the 3(p21–p26) interval,[15,23–26] although no specific candidate genes from this region had previously been identified. Our association study confirms the results of these genome-wide scans and further shows that *PPARG* is a candidate susceptibility gene within this interval. In addition, our results in the SAMP1/Fc model suggest that the critical factor in regulation of disease susceptibility may be the level of *Pparg* expression and activation in the crypts of the small intestine.

Whether the chromosome 6 susceptibility locus initially identified in our (C57BL/6 × SAMP1/Fc)F_2 cross[3] also maps to *Pparg* still is unclear. In selective genotyping across the *Pparg* locus, we have identified only a single base that distinguishes B6 from SAMP1/Fc, and the putative regulatory region that binds the Cdx/Nkx transcription factors is identical in the 2 strains. However, epithelial expression of Pparγ in the small intestine of B6 mice falls in-between the levels in SAMP1/Fc and AKR mice. This suggests that other long-range regulators of expression, as yet unidentified, may differ between SAMP1/Fc and B6 mice. Congenic mouse strains, which will allow fine-mapping of the B6-derived protective locus and further study of the regulation of the B6 allele of *Pparg*, currently are being developed.

Pparγ is a ligand-activated transcription factor that plays a central role in adipocyte differentiation and insulin sensitivity,[27,28] but this molecule also is central to signal transduction pathways involved in controlling inflammatory responses. Inhibition of proinflammatory transcriptional regulators such as nuclear factor κ B (NF-κB), Jun N-terminal kinase, and p38 all have been linked to PPARγ activity in vitro in cells of hematopoietic lineages (reviewed in [29]). Expression of the Pparγ1 isoform is found in most epithelial tissues, and earlier studies suggested that expression in the colon was much higher than that in small intestine.[18] A role for Pparγ in colonic epithelial differentiation was proposed, based on the finding that exposure of the colonic epithelium to butyrate and other Pparγ agonists promotes cell differentiation and tightening of epithelial junctions in vitro.[30] Recent studies also have shown that it is expressed at decreased levels in colonic tissue from ulcerative colitis patients.[31] Although the decreased expression in ulcerative colitis simply could reflect the disordered epithelial cell differentiation near sites of inflammation, earlier data from several animal models suggest that Pparγ activity may regulate susceptibility to the development of colitis in mice.

In one study, 2 Pparγ agonists, troglitazone and rosiglitazone, prevented the colonic inflammation associated with dextran sodium sulfate administration in mice.[32] However, in contrast to the decreased expression of epithelial PPARγ seen in humans with ulcerative colitis, severely affected control mice in these studies continued to express high levels of Pparγ in colonic epithelium. In a second study, pretreatment with ligands for both Pparγ and its binding partner retinoid X receptor decreased the severity of experimental colitis induced with 2,4,6-trinitrobenzene sulfonic acid,[33] and mice heterozygous for a null allele of *Pparg* were found to be significantly more susceptible to disease induction in this model.[33] Despite these suggestive findings in humans and mouse models, the one published study attempting to link *PPARG* to susceptibility to ulcerative colitis in humans failed to show evidence for genetic association.[31]

In addition to their failure to detect evidence for genetic association in human ulcerative colitis, Dubuquoy et al[31] were unable to detect differences in colonic expression of this molecule between individuals with CD and control subjects and suggested that the effects of this molecule might be important only in inflammatory disorders of the colon. Consistent with their negative data, we also found no differences between Pparγ expression in the colonic tissue of ileitis-prone SAMP1/Fc mice. However, we have shown major differences in both *Pparg* messenger RNA levels and localization of Pparγ expression in small intestine from the same mice at an age that precedes histologic evidence of intestinal inflammation (4 weeks). Together, the data from mice suggest that absence of *Pparg* expression actually may play a role in targeting inflammatory responses to a particular segment of the intestine, although definitive tests using tissue-specific deletion of *Pparg* have not yet been performed.

Su et al[32] reported that PPARγ agonist treatment decreases interleukin-1β–induced proinflammatory cytokine production in Caco-2 cells through an IκBα-dependent mechanism. More recent studies using Caco-2 cells suggest that PPARγ also may regulate NF-κB activity through a novel mechanism, resulting in coordinated nuclear export of both RelA and PPARγ. Kelly et al[34] showed that infection with *Salmonella enteriditis* produced a rapid induction of interleukin-1β as well as interleukin-8. However, in cells co-infected with both *S enteriditis* and a commensal organism (*Bacteroides thetaiotaomicron*),

PPARγ-dependent nuclear export of NF-κB complexes resulted in decreased production of the proinflammatory cytokines tumor necrosis factor α and interleukin-8, as well as decreased inflammation in vivo. Together, these studies suggest a direct link between epithelial PPARγ expression and the establishment of a proinflammatory or anti-inflammatory environment in the intestine in the presence of mixed microbial flora.

The intracellular pathogen receptor *N0D2/CARD15*, the only other human susceptibility gene clearly identified to date,[35] also appears to function by regulation of NF-κB activity. Although the original reports suggested that NOD2 expression is largely restricted to macrophages and monocytes, more recently it has been shown to be expressed at high levels in small intestinal crypt epithelium.[36] Co-expression of both NOD2 and PPARγ in the same epithelial compartment suggests that these 2 molecules may operate together, through control of NF-κB signaling, to regulate proinflammatory responses by the critical regenerative compartment in the small intestinal crypts to challenge by intracellular microorganisms. Specific mutations in *N0D2* gene are predicted to account for genetic risk in only a small subset of affected individuals. We postulate that PPARγ, through regulation of NF-κB activity, may contribute to genetic risk in the majority of individuals with CD who do not carry causative mutations in *N0D2*.

Although treatment with PPARγ agonists was effective in preventing inflammatory damage in the dextran sodium sulfate and 2,4,6-trinitrobenzene sulfonic acid mouse models of chemically induced colitis, other studies clearly have shown that they are ineffective in treatment of established inflammation in the same models once expression of Pparγ disappears.[37] Our data suggest that the low levels of residual PPARγ expression in epithelial crypts found in (C57BL/6 × SAMP1/Fc)F_1 mice provide an adequate therapeutic target for nonphysiologic, pharmacologic activation in chronic, spontaneous disease. However, our observation that rosiglitazone actually may increase the severity of ileitis in SAMP1/Fc mice suggests that treatment of individuals with no expression of Pparγ in the intestinal crypts actually may worsen disease, perhaps through activation in hematopoietic cells where expression levels appear not to differ between the strains in our study. This finding suggests caution in the use of PPARγ agonists to treat inflammatory bowel disease in the absence of documented expression in the epithelial crypts.

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Address requests for reprints to: Marcia McDuffie, MD, Departments of Microbiology and Internal Medicine, University of Virginia School of Medicine, MR-4, Box 801390, Charlottesville, Virginia 22908. e-mail: mjm7e@virginia.edu; fax: (434) 924-1221.

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