Characterization of Bglu3, a mouse fasting glucose locus, and identification of Apcs as an underlying candidate gene

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Departments of 1Radiology and Medical Imaging, 3Microbiology, and 4Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia; and 5School of Chemical Engineering and Technology, Tianjin University, Tianjin, China

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Li J, Lu Z, Wang Q, Su Z, Bao Y, Shi W. Characterization of Bglu3, a mouse fasting glucose locus, and identification of Apcs as an underlying candidate gene. Physiol Genomics 44: 345–351, 2012. First published January 24, 2012; doi:10.1152/physiogenomics.00087.2011.—Bglu3 is a quantitative trait locus for fasting glucose on distal chromosome 1 identified in an intercross between C57BL/6 (B6) and C3H/HeJ (C3H) apolipoprotein E-deficient (apoE−/−) mice. This locus was subsequently replicated in two separate mouse intercrosses. The objective of this study was to characterize Bglu3 through construction and analysis of a congenic strain and identify underlying candidate genes. Congenic mice were constructed by introgressing a genomic region harboring Bglu3 from C3H.apoE−/− into B6.apoE−/− mice. Mice were started with a Western diet at 6 wk of age and maintained on the diet for 12 wk. Gene expression in the liver was analyzed by microarrays. Congenic mice had significantly higher fasting glucose levels and developed more significant glucose intolerance compared with B6.apoE−/− mice on the Western diet. Microarray analysis revealed 336 genes to be differentially expressed in the liver of congenic mice. Further pathway analysis suggested a role for acute phase response signaling in regulating glucose intolerance. Apcs, encoding an acute phase response protein serum amyloid P (SAP), is located underneath the linkage peak of Bglu3. Multiple single nucleotide polymorphisms between B6 and C3H mice were detected within and surrounding Apcs. Apcs expression in the liver was significantly higher in congenic and C3H mice compared with B6 mice. The Western diet consumption led to a gradual rise in plasma SAP levels, which was accompanied by rising fasting glucose in both B6 and C3H apoE−/− mice. Expression of C3H Apcs in B6.apoE−/− mice aggravated glucose intolerance. Bglu3 is confirmed to be a locus affecting diabetes susceptibility, and Apcs is a probable candidate gene.

HYPERGLYCEMIA AND GLUCOSE intolerance are the defining characteristics of Type 2 diabetes in humans, and dyslipidemia often precedes or accompanies the disorder. One commonly used mouse model of dyslipidemia is the apolipoprotein E-deficient (apoE−/−) mouse, which exhibits the typical features of dyslipidemia seen in humans, including elevations in non-HDL cholesterol and triglyceride levels and reductions in HDL cholesterol levels (19, 31). We have found that apoE−/− mice on either C57BL/6 (B6) or C3H/HeJ (C3H) genetic background develop significant hyperglycemia and severe dyslipidemia when fed a Western-type diet (22). Using an intercross derived from B6.apoE−/− and C3H.apoE−/− mice, we identified a significant locus on distal chromosome 1, named Bglu3, contributing to variations in fasting glucose levels (22). This locus has been replicated in two intercrosses, including one between New Zealand obese (NZO) and B6 mice (25) and one between B6 and BALB/c apoE−/− mice (32). The human syntenic region of Bglu3 (156.1–188.6 Mb) corresponds to chromosome 1q21–q23, a region that has shown strong linkage to Type 2 diabetes in a number of populations including Pima Indians (7), Utah Caucasians (3), French Caucasians (24), British Caucasians (26), and Chinese (27). However, the specific diabetes susceptibility genes remain to be identified.

Serum amyloid P (SAP) is a member of the pentraxin family that also includes C-reactive protein and long pentraxin 3. The pentraxins play an important role in inflammation and innate immunity as they are capable of recognizing microbial pathogens, activating complement, and stimulating the uptake of pathogens by phagocytes (4). Studies have shown that activation of the inflammation cascade leads to beta-cell damage, insulin resistance, and the vascular complications of diabetes (6). SAP is also a vital component of extracellular matrix, in particular glomerular basement membrane, and a component of amyloid deposits in Alzheimer’s disease (1) and Type 2 diabetes (5). We have found that higher plasma SAP levels are associated with more severe hyperglycemia in the intercross that led to the identification of Bglu3 (22). Apcs (174.8 Mb), which encodes SAP, is a positional candidate gene located underneath the linkage peak of Bglu3. A human case-cohort study has shown that higher SAP levels are correlated with fasting glucose, fasting insulin, and body mass index (9). In the present study, we therefore evaluated Apcs as a potential candidate gene of Bglu3 affecting diabetes susceptibility in mice.

RESEARCH DESIGN AND METHODS

Mice. B6.apoE−/− mice were purchased from the Jackson Laboratory, and C3H.apoE−/− mice were generated in our laboratory. Chromosome 1 congenic strain (B6.C3H-Chr1.apoE−/−) were generated by introgressing a chromosomal segment harboring Bglu3 (149–187 Mb) from the donor strain C3H.apoE−/− into B6.apoE−/− recipient mice using the standard congenic breeding strategy (20). Microsatellite markers, D1Mit442 (125.8 Mb), D1Mit102 (149.1 Mb), D1Mit267 (157.9 Mb), D1Mit425 (158.5 Mb), D1Mit15 (170.2 Mb), D1Mit111 (170.9 Mb), D1Mit205 (172.3 Mb), D1Mit356 (174.8 Mb), D1Mit206 (174.9 Mb), D1Mit221 (187 Mb), and D1Mit117 (191.7 Mb), were used to evaluate introgression of the congenic segment. The proximal end of the congenic segment is between D1Mit442 and D1Mit102, and the distal end is between D1Mit221 and D1Mit117. The absence of C3H DNA in other chromosomal regions of congenic mice was confirmed by typing for microsatellite markers roughly spaced at 15 cM throughout the entire genome.

To generate transgenic mice, a clone containing C3H Apcs gene in the pTARBAC2.1 vector was purchased from the Pieter De Jong’s labora-
Glucose tolerance test (GTT) and insulin tolerance test (ITT) for congenic mice harboring the C3H allele of Bglu3 and B6.apoE<sup>−/−</sup> control mice fed a Western diet. For GTT, mice were fasted overnight and then injected with glucose (1 g/kg ip) ITT was performed by an injection of insulin (0.75 U/kg ip) to nonfasted mice. Blood glucose concentrations were determined with a glucometer using blood taken from cut tail tips at the indicated time points. Values are means ± SE of 18–24 mice. *P < 0.05 vs. B6.apoE<sup>−/−</sup> mice at a specific time point, and †P < 0.05 vs. B6.apoE<sup>−/−</sup> mice over the entire curve.

Fig. 1. Glucose tolerance test (GTT) and insulin tolerance test (ITT) for congenic mice harboring the C3H allele of Bglu3 and B6.apoE<sup>−/−</sup> control mice fed a Western diet. For GTT, mice were fasted overnight and then injected with glucose (1 g/kg ip) ITT was performed by an injection of insulin (0.75 U/kg ip) to nonfasted mice. Blood glucose concentrations were determined with a glucometer using blood taken from cut tail tips at the indicated time points. Values are means ± SE of 18–24 mice. *P < 0.05 vs. B6.apoE<sup>−/−</sup> mice at a specific time point, and †P < 0.05 vs. B6.apoE<sup>−/−</sup> mice over the entire curve.

Glucose tolerance test and insulin tolerance test. Glucose tolerance test (GTT) was performed as described by McDuffie et al. (15). In brief, mice were fasted overnight and then subjected to an intraperitoneal injection of glucose (1 g/kg). Blood glucose levels were measured with an UltraTouch glucometer using the whole blood taken from cut tail tips immediately before and at 10, 20, 30, 60, 90, and 120 min after the injection of glucose. Insulin tolerance test (ITT) was performed on nonfasted mice by intraperitoneal injection of 0.75 U/kg of insulin. Blood glucose was also monitored with an UltraTouch glucometer immediately before and at 15, 30, 45, and 60 min after insulin injection.

Measurements of plasma glucose and SAP. All blood samples were collected from overnight fasted mice through retro-orbital vein puncture with the animals under isoflurane anesthesia. Plasma glucose was measured with a Sigma glucose (HK) assay kit. Plasma SAP was measured with a commercial ELISA kit (Immunology Consultants Laboratory, Newberg, OR).

Real-time PCR analysis. Real-time PCR was carried out to quantitate hepatic expression of Apcs in mice, as we previously described (28). β-Actin or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was amplified simultaneously in a separate set of tubes under the same condition and used as controls.

Statistical analysis. Values were expressed as means ± SE, with n indicating the number of animals. ANOVA was used to test for statistical significance between strains in blood glucose levels over time during the GTT or ITT. The Student t-test was used when only

Fig. 2. Canonical pathway analysis of differentially expressed genes in the liver of congenic and B6.apoE<sup>−/−</sup> mice. The top 11 significantly enriched pathways are shown. The log<sub>2</sub>-fold enrichment was calculated using the right-tailed Fisher’s exact test. The flat yellow line on the plot represents significance threshold for all pathways, and the curved yellow line represents −log P value of differentially expressed genes to the total number of genes in a pathway. Only significantly differentially expressed genes that were not labeled as “absent” for both congenic and B6.apoE<sup>−/−</sup> mice in the dataset were subject to pathway analysis.
two means were compared. Differences were considered statistically significant at $P < 0.05$.

RESULTS

**Glucose intolerance of congenic mice.** A congenic line carrying the C3H allele of Bglu3 was generated by using the classical congenic breeding protocol. The proximal boundary of the congenic segment is between D1Mit442 and D1Mit102, and the distal boundary lies between D1Mit221 and D1Mit117. Female congenic and B6.apoE$^{-/-}$ mice were switched onto a Western diet at 6 wk of age and maintained on the diet for 12 wk. To perform GTT, mice were fasted overnight and then injected intraperitoneally with glucose. Blood glucose was measured with a glucometer using whole blood taken from cut tail tips. Congenic mice developed more significant glucose intolerance compared with B6.apoE$^{-/-}$ mice (Fig. 1). Blood glucose levels were significantly higher in the congenics over the entire time course of GTT ($P < 0.001$) and also at 10, 20, 90, and 120 min ($P < 0.05$) after glucose injection. In addition, fasting blood glucose levels at the baseline (0 min) were significantly higher in the congenics than in the control mice (136.2 ± 8.5 vs. 113.6 ± 4.8 mg/dl, $P = 0.028$).

ITT was performed on nonfasted mice. In response to insulin, congenic mice showed a larger decrease in blood glucose levels over the entire testing period compared with the control mice, although the difference did not reach statistical significance (Fig, 2, $P = 0.073$). At the 45th and 60th min after insulin injection, blood glucose levels were significantly lower in the congenics than in the control mice ($P < 0.05$). The nonfasting blood glucose level before the injection of insulin was also higher in the congenics (208.6 ± 8.2 vs. 190.4 ± 5.4 mg/dl), although the difference was not statistically significant ($P = 0.075$).

**Global gene expression analysis.** Gene expression in the liver was compared between congenic and B6.apoE$^{-/-}$ mice when fed the Western diet. 336 genes were found to be differentially expressed between them (Supplemental Table S1).1 Of these, 12 genes were located within the confidence interval of Bglu3 (156–188 Mb). Apcs, Cd48, Fcgr3, Fmo3, Ifi202b, Ifi203, Ifi205, Kcnj10, and Tor3a were upregulated in congenics while Grem2, Ivns1abp, and Pex19 were downregulated.

All differentially expressed genes were then analyzed using the Ingenuity Pathway Analysis software to identify underlying biological pathways leading to phenotypical differences between congenic and control mice. Systemic lupus erythematosus signaling and acute phase response signaling are the two most significantly enriched pathways (Fig. 2). Among the 14

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1 The online version of this article contains supplemental material.
differentially expressed genes in the systemic lupus erythematosus signaling pathway and the 15 differentially expressed genes in the acute phase response signaling pathway. Apcs is the only gene that is located within the confidence interval of Bglu3 (Supplementary Table S2).

Apcs sequence variation. The entire Apcs gene, including its two exons, one 110-bp intron, and proximal promoter region, was sequenced for the B6 and C3H strains (Fig. 3). We identified nine single nucleotide polymorphisms (SNPs), including −182C>T, −43G>A, and −32T>C in the promoter region, 2C>G, 58A>G, and 76A>G in exon 1, 138A>T in intron, 654C>T and 912A>G in exon 2. 76A>G and 654C>T were the only two SNPs located in the coding region, but they resulted in no changes in amino acids of the protein product. The T→C SNP at −32 bp upstream of Apcs in C3H led to the loss of a NKX-2 binding site (CTTCATTG) and at the same time created an NIT2 binding site (TTATC). The other two SNPs in the promoter region were not within transcription factor binding sites.

Apcs expression in congenic and two progenitor strains. Given the multiple SNPs detected in the promoter region, Apcs was examined for its expression in the liver of B6.apoE−/−, C3H.apoE−/−, and congenic mice by quantitative real-time PCR and also by ELISA to measure plasma SAP levels (Fig. 4). In agreement with our previous observation (22), C3H.apoE−/− mice expressed much more SAP than B6.apoE−/− mice. The ratio of Apcs to β-actin mRNA copy numbers was 3.0 ± 1.0 for C3H mice, but it was 0.40 ± 0.09 for B6 mice (P = 0.12). Plasma SAP level was nearly threefold as high in C3H mice as in B6 mice (722.5 ± 43.3 vs. 277.3 ± 26.0 μg/ml, P = 0.0003). Like C3H, congenic mice expressed much more SAP than B6.apoE−/− mice. The ratio of Apcs to GAPDH mRNA copy numbers was 1.69 ± 0.28 for the congenics, significantly higher than the ratio of 0.60 ± 0.16 in B6.apoE−/− mice (P = 0.024). Plasma SAP levels were approximately threefold as high in congenics as in B6.apoE−/− mice (807.5 ± 44.6 vs. 243.8 ± 34.4 μg/ml, P = 3.5 × 10−7).

Diet-induced rises of plasma glucose and SAP levels. Both B6.apoE−/− and C3H.apoE−/− mice showed a slow rise in both plasma SAP and glucose levels over the 12 wk feeding period on the Western diet (Fig. 5). The concordant elevations of both SAP and glucose were especially obvious for B6.apoE−/− mice. SAP levels were much higher in C3H.apoE−/− than in B6.apoE−/− mice at all the time points examined. The difference was about sixfold on the chow diet (648.2 ± 34.0 vs. 127.8 ± 41.8 μg/ml, P = 0.00007) and was diminished to about threefold at the end of the Western diet feeding (722.3 ± 43.3 vs. 277.3 ± 22.5 μg/ml, P = 0.00003). Plasma glucose levels were also higher in C3H.apoE−/− than in B6.apoE−/− mice before the initiation of (163.6 ± 7.9 vs. 144.0 ± 7.4 mg/dl) and at the end of the Western diet (311.6 ± 25.5 vs. 242.9 ± 14.9 mg/dl), although the differences were not statistically significant (P = 0.14 and P = 0.18, respectively).

Characterization of transgenic mice. To directly evaluate the role of Apcs in glucose metabolism, we constructed transgenic mice expressing the C3H Apcs. Transgenic founders with the
B6D2 genetic background were serially backcrossed with B6.apoE−/− mice for more than six generations, and the resulting mice were kept as heterozygotes for the transgene. Transgenic mice exhibited an approximately twofold increase in Apcs mRNA expression levels in the liver over nontransgenic mice (0.259 ± 0.017 vs. 0.159 ± 0.015, P = 0.0003) (Fig. 6). Intraperitoneal GTT and ITT were performed after mice had been fed the Western diet for 11 and 12 wk, respectively. Blood glucose was monitored with an UltraTouch glucometer using blood taken from cut tail tips. As shown in Fig. 7, blood glucose levels during the entire time course of GTT were significantly higher in transgenic mice than nontransgenic littermates (P = 0.0008). The difference was more obvious at 60 min after glucose injection (P = 0.0076). In contrast, the insulin-induced fall of blood glucose levels under the nonfasting condition was comparable between the two groups (P = 0.94). In addition, there were no significant differences between transgenic and nontransgenic mice at the baseline (0 min) in both fasting (113.2 ± 9.1 vs. 118.6 ± 11.9 mg/dl) and nonfasting glucose levels (190.9 ± 7.8 vs. 196.5 ± 15.9 mg/dl).

DISCUSSION

Bglu3 is a major quantitative trait locus (QTL) for fasting glucose originally identified in an intercross between B6.apoE−/− and C3H.apoE−/− mice when mice have developed significant hyperglycemia on a Western diet (22). The C3H allele of Bglu3 confers an increased plasma glucose level on the Western diet, whereas the B6 allele reduces it. In the present study, we confirmed the existence of Bglu3 through an analysis of a congenic line that carried the C3H allele on the B6.apoE−/− genetic background. Congenic mice exhibited a significant increase in fasting glucose levels and glucose intolerance on the Western diet. The increase in fasting blood glucose was >20 mg/dl, and the peak elevation in blood glucose levels on the GTT approached 50 mg/dl. With regard to the polygenetic control of blood glucose levels, this effect from Bglu3 appeared to be quite significant.

We previously have observed a significant association of plasma SAP with fasting glucose in an intercross deficient in apoE (22). As the liver is the primary source of plasma SAP and the most important organ in maintaining glucose homeostasis, we examined gene expression in this organ of congenic and B6.apoE−/− mice by microarrays. A number of genes involved in the immune response were found to be up- or downregulated in the congenic strain. However, among them only Apcs and few others are located in the congenic region. Since congenics differ from their background strain only in the congenic region, those differentially expressed genes should be those in the congenic region or secondary consequences of gene differences in the congenic region. Thus, Apcs may be involved in regulating the expression of genes participating in the immune response.

Using combined congenic and gene expression profiling strategies, we were able to pinpoint Apcs as the most probable candidate gene of Bglu3. SAP was upregulated in the liver of congenic mice compared with their background strain as detected by the microarrays, and this result was confirmed by real-time PCR and ELISA. Genetic studies of recombinant inbred strains derived from B6 × DBA and B6 × C3H mice and of an intercross between B6 × C3H apoE−/− mice indicate that the baseline production of SAP is largely genetically determined by the Apcs locus (14, 16, 22). Itoh et al. (8) have illustrated that mouse strain-dependent variation in SAP expression is controlled at the transcriptional level. Our present observation that the congenic strain carrying the C3H Apcs gene in the B6 background had almost the same SAP level as C3H mice has confirmed the previous conclusion on the cis control of SAP production. There were multiple SNPs between the B6 and C3H strains within and surrounding Apcs. One SNP in the proximal promoter region of Apcs in C3H has led to a loss in the NXX-2 binding site and a creation of the NIT2 binding site. Itoh et al. (8) have suggested that a transcription factor with suppressor activity encoded by a gene linked to Apcs be involved in regulating Apcs expression. We speculate

![Fig. 6. Real-time PCR analysis of Apcs expression in the liver of transgenic and nontransgenic mice when fed a Western diet. Results are expressed as a ratio of Apcs to GAPDH in real-time PCR cycle threshold values. Values are means ± SE of 4 transgenic or 5 nontransgenic mice. *P < 0.05 vs. control.](image-url)

![Fig. 7. GTT and ITT for transgenic with C3H Apcs and nontransgenic littermates fed a Western diet. Transgenic mice were backcrossed with B6.apoE−/− mice for >6 generations. For GTT, mice were fasted overnight and injected with glucose (1 g/kg ip). ITT was performed on nonfasted mice by injection with insulin (0.75 U/kg). Blood glucose concentrations were determined with a glucometer using blood collected from cut tail tips at the indicated time points. Values are means ± SE of 8–12 mice. *P < 0.05 vs. nontransgenic littermates at a specific time point, and +P < 0.05 vs. nontransgenic mice over the entire curve.](image-url)
that the lost NKK-2 binding site may contribute to the increased SAP expression in C3H mice.

Although higher plasma levels of SAP have been associated with hyperglycemia in both mouse and human (9, 22), it has not been established whether Apcs actually affects diabetes susceptibility. The present study provides strong evidence supporting Apcs to be a diabetes susceptibility gene. Indeed, C3H.ApoE<sup>−/−</sup> mice expressing more SAP exhibited higher plasma glucose levels on the Western diet than B6.ApoE<sup>−/−</sup> mice expressing less SAP. Accordingly, F<sub>2</sub> mice with the C3H allele at the Apcs locus had higher fasting plasma levels than those with the B6 allele (data not shown). As there is a possibility that a different linked gene may contribute to the observed association in the F<sub>2</sub> population due to linkage disequilibrium, we constructed transgenic mice with the C3H Apcs. The present finding that transgenic mice developed more severely impaired glucose intolerance demonstrates an etiologic role for Apcs in diabetes. Although the effect of Apcs on glucose intolerance was small, given the fact that Type 2 diabetes is a polygenic disease, this contribution appears to be clinically relevant.

A high diet intake led to a time-dependent rise in plasma SAP levels, accompanied by a rise in fasting glucose levels, in the two apoE<sup>−/−</sup> parental strains. The increase in plasma SAP levels observed in the apoE<sup>−/−</sup> mice on the high-fat diet was much smaller than that observed during an acute inflammatory response but is similar in magnitude to that observed in chronic inflammation in which circulating SAP contributes to the pathogenesis of secondary amyloidosis (17) (29). Thus, the modest elevations of SAP appear to reflect a chronic inflammatory response that is pathologically relevant to the clinical situation. We and others have observed that the consumption of a high-fat diet contributes to modest elevations of circulating inflammatory cytokines, such as VCAM-1, P-selectin, IL-1, IL-6, and TNF-α (2, 10, 12, 23). IL-1 and IL-6 have been shown to induce the production of SAP by hepatocytes (13). Thus, increased SAP levels could also be secondary to the increases of other cytokines induced by the diet.

Although the congenic segment also harbors a QTL for body weight (22), we have found no increases in body weight of the congenic strain (data not shown). One probable explanation is that diabetes, once developed, causes weight loss. Insulin is a key regulator that let nutrients into cells. For energy, the body will burn off fat cells and subsequently cause weight loss in diabetes due to the insufficiency of insulin. Impaired insulin secretion is responsible for the development of diabetes in B6.ApoE<sup>−/−</sup> mice (11). As the congenics developed more severe diabetic, the genetic effect from the chromosome 1 QTL on body weight was probably overridden by the effect from diabetes.

The demonstration that genetic variation of Apcs contributes to diabetes susceptibility provides clear evidence supporting the involvement of the innate immunity in the pathogenesis of the disorder. Recent findings from genome-wide association studies suggest that this is the case for humans as well as mice. The findings that CDKAL1, GCKR, and THADA are associated with Type 2 diabetes susceptibility in humans (18, 21, 30) supports this possibility. Female mice were used for the present study because the locus of Bglu3 was originally identified in female F<sub>25</sub> derived from an intercross between B6.ApoE<sup>−/−</sup> and C3H.ApoE<sup>−/−</sup> mice. However, the present findings may not be extrapolated to male mice since they were not studied.

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The accession number of the C3H/HeJ Apcs gene sequence in the NCBI GenBank is HQ283363. The accession number of the microarray data in the NCBI GEO database is GSE29151.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

J.L. and W.S. conception and design of research; J.L., Z.L., Q.W., Z.S., and W.S. performed experiments; J.L., Q.W., Y.B., and W.S. analyzed data; J.L., Y.B., and W.S. interpreted results of experiments; J.L., Q.W., and W.S. prepared figures; J.L., Z.L., Q.W., Z.S., Y.B., and W.S. approved final version of manuscript; W.S. drafted manuscript; W.S. edited and revised manuscript.

REFERENCES


