

Haplotype Structures and Large-Scale Association Testing of the 5' AMP-Activated Protein Kinase Genes *PRKAA2*, *PRKAB1*, and *PRKAB2* With Type 2 Diabetes

Maria W. Sun,^{1,2} Jennifer Y. Lee,^{1,2} Paul I.W. de Bakker,^{1,2,3} Noël P. Burttt,² Peter Almgren,⁴ Lennart Råstam,⁵ Tiinamaija Tuomi,⁶ Daniel Gaudet,⁷ Mark J. Daly,^{2,8} Joel N. Hirschhorn,^{2,3,9} David Altshuler,^{1,2,3,8,10} Leif Groop,^{4,6} and Jose C. Florez^{1,2,8,10}

AMP-activated protein kinase (AMPK) is a key molecular regulator of cellular metabolism, and its activity is induced by both metformin and thiazolidinedione antidiabetic medications. It has therefore been proposed both as a putative agent in the pathophysiology of type 2 diabetes and as a valid target for therapeutic intervention. Thus, the genes that encode the various AMPK subunits are intriguing candidates for the inherited basis of type 2 diabetes. We therefore set out to test for the association of common variants in the genes that encode three selected AMPK subunits with type 2 diabetes and related phenotypes. Of the seven genes that encode AMPK isoforms, we initially chose *PRKAA2*, *PRKAB1*, and *PRKAB2* because of their higher prior probability of association with type 2 diabetes, based on previous reports of genetic linkage, functional molecular studies, expression patterns, and pharmacological evidence. We determined their haplotype structure, selected a subset of tag single nucleotide polymorphisms that comprehensively capture the extent of common genetic variation in these genes, and genotyped them in family-based and case/control samples comprising 4,206 individuals. Analysis of single-marker and multi-marker tests revealed no association with type 2 diabetes, fasting

plasma glucose, or insulin sensitivity. Several nominal associations of variants in *PRKAA2* and *PRKAB1* with BMI appear to be consistent with statistical noise. *Diabetes* 55:849–855, 2006

Type 2 diabetes arises from the complex interplay of various pathophysiologic mechanisms involving peripheral insulin resistance and relative insulin insufficiency. The final expression of the diabetic phenotype is strongly influenced by inheritance; however, with the exception of rare monogenic forms of diabetes, common type 2 diabetes is thought to have a polygenic architecture (1). The two most widely reproduced associations of common variants with type 2 diabetes, the P12A polymorphism in the peroxisome proliferator-activated receptor (PPAR)- γ (encoded by *PPARG*) and the E23K polymorphism in the islet ATP-activated potassium channel Kir6.2 (encoded by *KCNJ11*), occur in genes that are targets for antidiabetic medications (2).

AMP-activated protein kinase (AMPK), a central cellular energy regulator, has recently emerged as a primary candidate for a role in metabolic dysfunction (3). This highly conserved heterotrimer consists of an α catalytic subunit and β and γ regulatory subunits, each of which has more than one isoform. The activation of AMPK by the depletion in cellular energy levels results in a host of metabolic effects ranging from mitochondrial biogenesis to improved insulin sensitivity (4–6), leading to increases in glucose availability and fatty acid oxidation (7,8). Enhanced AMPK activity in low-energy states is thought to be due to both transcriptional and post-translational mechanisms. AMPK activity is induced in human muscle as a result of moderate- to high-intensity exercise, with corresponding increases in phosphorylation and deactivation of acetyl-CoA carboxylase- β (a key enzyme in fatty acid synthesis) and nuclear translocation of the catalytic $\alpha 2$ subunit (9–11). Interestingly, acute exercise, which normalizes GLUT4 translocation and glucose uptake in patients with type 2 diabetes, causes a normal (approximately threefold) increase in $\alpha 2$ activity in these patients (12).

The $\alpha 2$ catalytic subunit, encoded by *PRKAA2* in chromosome 1p31, is phosphorylated under stimulation by the widely used antidiabetic drug metformin (13–16). Although this relationship is well established, the precise mechanism of activation has yet to be elucidated. It does not appear to act through ATP depletion, nor through direct phosphorylation of the upstream kinase LKB1 (17,18).

From the ¹Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts; the ²Program in Medical and Population Genetics, Broad Institute of Harvard and MIT, Cambridge, Massachusetts; the ³Department of Genetics, Harvard Medical School, Boston, Massachusetts; the ⁴Department of Endocrinology, University Hospital MAS, Lund University, Malmö, Sweden; the ⁵Department of Clinical Science, University Hospital MAS, Lund University, Malmö, Sweden; the ⁶Department of Medicine, Helsinki University Central Hospital, Folkhalsan Genetic Institute, Folkhalsan Research Center and Research Program for Molecular Medicine, University of Helsinki, Helsinki, Finland; the ⁷University of Montreal Community Genomic Center, Chicoutimi Hospital, Quebec, Canada; the ⁸Department of Medicine, Massachusetts General Hospital, Boston, Massachusetts; the ⁹Divisions of Genetics and Endocrinology, Children's Hospital, Boston, Massachusetts; and the ¹⁰Department of Medicine, Harvard Medical School, Boston, Massachusetts.

Address correspondence and reprint requests to Jose C. Florez, Diabetes Unit/Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114. E-mail: jcflorez@partners.org.

Received for publication 31 October 2005 and accepted in revised form 9 December 2005.

M.W.S. is currently affiliated with the University of California-Davis School of Medicine, Sacramento, California. J.Y.L. is currently affiliated with the Department of Systems Biology, Harvard Medical School and Division of Signal Transduction, Beth Israel Deaconess Medical Center, Boston, Massachusetts.

AMPK, AMP-activated protein kinase; GRR, genotype relative risk; ISI, insulin sensitivity index; LD, linkage disequilibrium; MAF, minor allele frequency; OGTT, oral glucose tolerance test; PPAR, peroxisome proliferator-activated receptor; SNP, single nucleotide polymorphism.

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

TABLE 1
Clinical characteristics of patient samples

	Sex (M/F)	Age (years)	BMI (kg/m ²)	Fasting plasma glucose (mmol/l)	HbA _{1c} (%)* or plasma glucose at 2-h OGTT (mmol/l)†
Scandinavian trios					
Affected probands	168/153	39 ± 9	27 ± 5	7.2 ± 2.6	8.5 ± 2.9†
Parents	236/236				
Sibships					
Diabetes/severe IGT sib	280/329	65 ± 10	29 ± 5	9.3 ± 3.3	14.3 ± 5.6†
NGT sib	275/305	62 ± 10	26 ± 3	5.4 ± 0.4	6.0 ± 1.1†
Scandinavian C/C					
Diabetes/severe IGT	252/219	60 ± 10	28 ± 5	9.8 ± 3.4	15.0 ± 5.3†
NGT	254/217	60 ± 10	27 ± 4	6.2 ± 1.8	6.8 ± 2.8†
Swedish C/C					
Diabetes/severe IGT	267/247	66 ± 12	28 ± 4	9.6 ± 2.9	6.5 ± 1.5*
NGT	267/247	66 ± 12	28 ± 4	5.5 ± 0.7	ND
Canadian C/C					
Diabetes	70/57	53 ± 8	29 ± 5	6.4 ± 1.8	12.8 ± 2.1†
NGT	70/57	52 ± 8	29 ± 4	5.1 ± 0.6	6.1 ± 1.1†

Data are means ± SD. Plasma glucose was measured at baseline (fasting) and 2 h after an OGTT. C/C, case/control; IGT, impaired glucose tolerance; ND, not determined; NGT, normal glucose tolerance. Severe IGT was defined as a 2-h OGTT blood glucose ≥8.5 but <10.0 mmol/l.

PRKAA2 is located under a type 2 diabetes linkage peak detected in a diabetic Japanese population (logarithm of odds [LOD] score 1.24, nominal $P = 0.014$) (19). Furthermore, in contrast to mice devoid of the $\alpha 1$ subunit, $\alpha 2$ subunit knockout mice display hyperglycemia and insulin resistance (20). Finally, muscle contraction and moderate-intensity exercise have consistently resulted in increased $\alpha 2$ activity (21).

PRKAB1, the gene that encodes the $\beta 1$ regulatory subunit, is located in chromosome 12q24.1, under the 12q24.31 type 2 diabetes linkage peak found in several populations (22–24). Additionally, it has been shown that mutations in phosphorylation and post-translational modification sites in $\beta 1$ affect levels of AMPK activity and/or its nuclear distribution (25). The $\beta 1$ subunit also binds either glycogen or glycogen-debranching enzyme, resulting in changes to overall AMPK complex activity (26,27).

PRKAB2, the gene that encodes the $\beta 2$ regulatory subunit, is located in 1q21.2, near the type 2 diabetes linkage peak found in Pima Indians (28), which is supported by studies in additional populations (29–31). The $\beta 2$ subunit is the predominant isoform found in human skeletal muscle, and it associates primarily with the $\alpha 2$ isoform (32,33). Expression studies conducted by the Genomics Institute of the Novartis Research Foundation also provide evidence of high expression of $\beta 2$ in human liver (online database, <http://symatlas.gnf.org/SymAtlas/>) (34).

Taken together, these various lines of evidence establish *PRKAA2*, *PRKAB1*, and *PRKAB2* as attractive candidate genes for increasing type 2 diabetes risk and influencing related physiological parameters. We therefore set out to characterize their haplotype structures and assess a comprehensive set of common variants in these genes for association with type 2 diabetes and intermediate phenotypes.

RESEARCH DESIGN AND METHODS

Haplotype structure. The haplotype structures of the $\alpha 2$, $\beta 1$, and $\beta 2$ genes were determined by selecting single nucleotide polymorphisms (SNPs) from the publicly available dbSNP database at regularly spaced intervals, and genotyping these in the HapMap reference panel from the CEPH (Centre d'Etude du Polymorphisme Humain) consisting of 30 parent-offspring trios

from Utah with European ancestry (CEU). This dataset was supplemented with genotypes available from the International HapMap Project (www.hapmap.org) for this same reference panel. For all genes, we extended our investigation of the haplotype structure both upstream and downstream of the gene until linkage disequilibrium (LD) decayed on both flanks, as indicated by the termination of a haplotype block according to the definition of Gabriel et al. (35). Accordingly, the region examined for *PRKAA2* extends for 100 kb, from 27 kb upstream of the transcription start site to 12 kb downstream of the 3' untranslated region; likewise for *PRKAB1* and *PRKAB2*, the regions analyzed cover 156 kb (from –84 to +74 kb) and 93 kb (from –38 to +35 kb), respectively. SNPs of <5% frequency, those that failed genotyping due to technical errors, or those that failed to meet Hardy-Weinberg equilibrium ($P < 0.01$) were removed from the haplotype structure and subsequent analyses.

Genotyping. Genotyping was performed as previously described, using primer extension of multiplex products with detection by MALDI-TOF (matrix-assisted laser desorption ionization–time of flight mass spectroscopy) on the Sequenom platform (35,36). Average genotyping success was 99.7% in the reference panel and 94% in the disease panel; our concordance rate, based on 1,281 duplicate comparisons, was 99.77%. Genotype counts for the various samples tested in this study are shown in online appendix Table 1 (available from <http://diabetes.diabetesjournals.org> as well as on our website http://genetics.mgh.harvard.edu/AltshulerWeb/publicationdata/Sun_AMPK.html).

Tagging methodology. Single- and multi-marker tests were identified using the software Tagger (37) (<http://www.broad.mit.edu/tagger>), such that these tests collectively capture all SNPs ≥5% frequency at a minimum r^2 of 0.8. We used the aggressive (multi-marker) tagging mode and constrained tag SNPs that appear in multi-marker tests to be in strong LD (LOD score >2.0). Phasing of chromosomes was performed by the weighted expectation-maximization algorithm incorporated into the software Haploview (38) (<http://www.broad.mit.edu/mpg/haploview/>) when possible and as previously described (39) for the discordant sibpairs.

Clinical samples. The diabetic samples are presented in Table 1 and have been described elsewhere (40,41). Briefly, they comprise 321 Scandinavian trios; 1,189 Scandinavian siblings discordant for type 2 diabetes; a Scandinavian case-control sample totaling 942 subjects individually matched for age, BMI, and geographic region; a case/control sample from Sweden totaling 1,028 subjects who were individually matched for sex, age, and BMI; and an individually matched case/control sample totaling 254 subjects from the Saguenay Lac–St. Jean region in Quebec, Canada. In the Scandinavian samples, case subjects included those with type 2 diabetes or severe impaired glucose tolerance, defined as a 2-h blood glucose of 8.5 to <10.0 mmol/l during an oral glucose tolerance test (OGTT). These samples have been validated by the replication of the two most widely reproduced associations in type 2 diabetes, *PPARG* P12A (40) and *KCNJ11* E23K (42), and by the overlap with other groups' findings in the promoter region of the hepatocyte nuclear factor 4 α (41).

Statistical analysis. Power calculations were performed using the program of Purcell et al. (43), available at <http://pnu.mgh.harvard.edu/~purcell/gpc/>. To examine the association of SNPs and haplotypes with type 2 diabetes, we

used simple χ^2 analysis in the case/control samples, the transmission disequilibrium test (44) in the diabetic trios, and the discordant allele test (45) in the sibpairs; the first two have been implemented in Haploview (<http://www.broad.mit.edu/mpg/haploview/>) for both single- and multi-marker association testing (38). In Haploview, possible ambiguity in haplotype assignments is accounted for by incorporating haplotype probabilities in the tests of disease association. The probability estimate of each multi-marker haplotype test was compared individually against all others; only haplotypes with frequencies >5% in the reference panel were examined. Results from the various samples were combined by Mantel-Haenszel meta-analysis of the odds ratios (ORs) (46); all *P* values are two-tailed. Homogeneity of ORs among study samples was tested using a Pearson χ^2 goodness-of-fit test, as previously described (46).

Quantitative trait comparisons. Plasma glucose was measured by a glucose oxidase method on a glucose analyzer (Beckman Instruments, Fullerton, CA). Insulin was measured by radioimmunoassay. A 75-g OGTT was performed in a subset of the control Scandinavian subjects ($n = 756$, 363 female). The whole-body insulin sensitivity index (ISI) was calculated as in Matsuda and DeFronzo (47). Nondiabetic individuals were sorted by their diploid genotypes at each locus; each most-likely inferred multi-marker haplotype test was compared against all other possibilities at the corresponding loci. Mean fasting plasma glucose, ISI, and BMI (the latter two after log transformation for non-normality) were compared by ANOVA across the three genotypic groups for each marker.

RESULTS

We initially selected 73 SNPs from the dbSNP database and genotyped them in the HapMap CEU panel. Of these SNPs, 37 passed our criteria for inclusion, including genotyping percentage >75%, Hardy-Weinberg equilibrium, and minor allele frequency (MAF) >5%. These SNPs were combined with genotypic data downloaded from the HapMap (CEU) to determine the structure of variation in *PRKAA2*, *PRKAB1*, and *PRKAB2*. Respectively, 40, 49, and 29 SNPs were used to characterize the haplotype structures of *PRKAA2*, *PRKAB1*, and *PRKAB2*, with an average spacing of one SNP every 2.5–3.3 kb; strong LD was noted for all three genes, with almost all variants contained within haplotype blocks as defined by Gabriel et al. (35). Detailed figures of each gene's haplotype structure can be found in the online appendix (supplementary Fig. 1A–C) as well as on our website (http://genetics.mgh.harvard.edu/AltshulerWeb/publicationdata/Sun_AMPK.html).

Our tagging procedure resulted in the selection of 22 tag SNPs in total, which collectively specify 22 single-marker and 18 multi-marker tests (9/6 for *PRKAA2*, 8/8 for *PRKAB1*, and 5/4 for *PRKAB2*). Thus, these 40 tests constitute the tests of association to the trait (both of themselves and of the other variants captured by them), which were performed in the disease samples (online appendix Fig. 1 and online appendix Table 2).

Assuming a type 2 diabetes prevalence of 8% and a multiplicative model, our power calculations demonstrated that our sample of 1,112 case/control pairs, 321 trios, and 1,189 discordant sibs had >90% power (at $P < 0.05$) to detect an association with type 2 diabetes for alleles or haplotypes of frequency $\geq 20\%$ if the genotype relative risk (GRR) was 1.2 or higher and for alleles or haplotypes of frequency $\geq 10\%$ if GRR was 1.3 or higher. For a GRR of 1.2, power was >70% for allele or haplotype frequencies of 10% and dropped to ~45% for allele or haplotype frequencies of 5%.

A meta-analysis of the association studies for the SNPs and multi-marker haplotypes in each of the genes is presented in Table 2. No heterogeneity was detected among subsamples. We observed no significant association with type 2 diabetes for any of these tests. Genotype counts for each subsample is available in the online appendix (supplementary Table 1) and on our website

(http://genetics.mgh.harvard.edu/AltshulerWeb/publicationdata/Sun_AMPK.html). After correcting for having tested multiple hypotheses by permutation, the nominal *P* values of 0.05 for rs2393550 in *PRKAB1* and 0.04 for test 38 in *PRKAB2* no longer reached statistical significance.

Because our case/control samples were matched for BMI, it is possible that overmatching in our case/control panels may have prevented us from detecting a true effect on risk of type 2 diabetes, if this effect was mediated through BMI. We therefore assessed whether BMI was associated with any of the 40 genotypic tests in our control sample. BMI comparisons across genotypic groups showed nominal *P* values <0.05 for several tests in *PRKAA2* and *PRKAB1*, although after correction by permutation testing, the best result did not retain empirical statistical significance (Table 3).

Given the role played by AMPK activation in glucose uptake and insulin resistance (6,48), we analyzed common variants in these genes for association with differences in fasting plasma glucose and in the ISI in the 756 control subjects for whom we had OGTT data. We found no significant differences in fasting plasma glucose for any of the 40 genotypic tests; the nominally significant differences in ISI observed for SNPs rs894467 and rs1890039 in *PRKAB2* were due to genotypic groups that only had two observations and therefore do not represent reliable findings (online appendix Table 3, also available at our website at http://genetics.mgh.harvard.edu/AltshulerWeb/publicationdata/Sun_AMPK.html).

DISCUSSION

We set out to test the hypothesis that genetic variation in the AMPK enzyme may contribute to the risk of type 2 diabetes. Based on the higher prior probability we ascribed to the $\alpha 2$, $\beta 1$, and $\beta 2$ subunits, we initially selected these three genes for investigation. In a sample comprising both case/control and family-based panels totaling 4,206 individuals, we were unable to document a significant association of a comprehensive set of common variants in the AMPK genes *PRKAA2*, *PRKAB1*, and *PRKAB2* with type 2 diabetes or two related intermediate traits.

Our negative results can have several explanations. First, genetic variation in AMPK may not contribute to the risk of type 2 diabetes. If functional variation is present, epigenetic factors (rather than common genetic variation) in AMPK may be responsible for its observed functional role in ameliorating derangements of glucose homeostasis in humans. It is also possible that genetic variants in AMPK, while influencing intermediate phenotypes, may not have an effect that is strong enough to impact diabetes risk; our inability to detect a major effect of AMPK variants on two related phenotypes (fasting plasma glucose and a measure of insulin sensitivity) argues against the latter explanation.

Second, our findings may represent false negative results. Although the power of a meta-analysis of five smaller subsamples is not equivalent to a well-designed association study of the same size, we note that no heterogeneity was detected among our subsamples and that the present design takes advantage of two family-based panels that are robust to population stratification. In addition, these same samples were adequate to detect the most commonly reproduced genetic associations with type 2 diabetes (40,42), both of which have a fairly modest effect on risk.

Third, genetic variation in AMPK genes that affects the

TABLE 2
Association of variants in *PRKAA2*, *PRKAB1*, and *PRKAB2* with type 2 diabetes

Gene	Test	SNP	Position	Alleles	MAF	OR	95% CI	P	
<i>PRKAA2</i>	1	rs2404987	56800626	T/C	0.48	1.05	0.95–1.17	0.31	
	2	rs6588640	56820444	G/A	0.07	0.92	0.74–1.14	0.43	
	3	rs2746343	56847835	C/T	0.16	0.99	0.86–1.15	0.93	
	4	rs857156	56875275	A/T	0.43	1.00	0.90–1.10	0.96	
	5	rs1342382	56889409	A/T	0.05	1.09	0.85–1.39	0.50	
	6	rs3738567	56890947	A/C	0.30	0.93	0.83–1.05	0.23	
	7	rs857142	56896486	C/A	0.32	0.96	0.86–1.07	0.45	
	8	rs10489617	56899028	C/G	0.12	1.00	0.82–1.22	0.97	
	9	rs2275732	56901814	T/C	0.05	1.20	0.89–1.61	0.23	
			Multi-marker tests		Hap	Freq	OR	95% CI	P
	10		rs6588640, rs2746343		A, T	0.05	1.08	0.87–1.34	0.47
	11		rs2404987, rs6588640, rs2746343		C, G, C	0.42	0.95	0.86–1.05	0.35
	12		rs2404987, rs2746343, rs3738567		T, T, C	0.12	1.06	0.90–1.25	0.50
	13		rs2746343, rs857156, rs1342382		C, T, A	0.44	0.99	0.89–1.11	0.93
	14		rs857156, rs1342382		A, A	0.38	1.01	0.91–1.12	0.83
15		rs857142, rs10489617		C, C	0.57	0.95	0.85–1.05	0.30	
Gene	Test	SNP	Position	Alleles	MAF	OR	95% CI	P	
<i>PRKAB1</i>	16	rs2015795	118499750	C/T	0.45	1.01	0.91–1.12	0.92	
	17	rs1541345	118546518	G/A	0.05	0.88	0.68–1.15	0.34	
	18	rs2393550	118560728	G/A	0.20	0.88	0.78–1.00	0.05	
	19	rs278143	118571734	A/G	0.35	1.03	0.91–1.16	0.64	
	20	rs278123	118587298	G/A	0.34	1.03	0.91–1.17	0.65	
	21	rs278124	118592151	A/G	0.19	0.92	0.79–1.07	0.29	
	22	rs2285595	118619128	A/G	0.50	0.92	0.83–1.02	0.10	
	23	rs278109	118634715	C/T	0.23	0.91	0.80–1.03	0.13	
			Multi-marker tests		Hap	Freq	OR	95% CI	P
	24		rs2015795, rs278123		C, G	0.23	1.11	0.99–1.24	0.07
	25		rs2015795, rs1541345, rs278123		T, G, G	0.42	0.99	0.87–1.12	0.88
	26		rs2015795, rs278143		C, A	0.23	1.05	0.94–1.17	0.43
	27		rs2393550, rs278124		A, G	0.19	1.07	0.93–1.22	0.36
	28		rs278124, rs278109		G, T	0.19	1.10	0.94–1.28	0.22
	29		rs2393550, rs278123		G, A	0.31	0.91	0.80–1.02	0.11
30		rs278123, rs2285595, rs278109		A, G, C	0.27	0.94	0.80–1.11	0.48	
31		rs2285595, rs278109		G, C	0.28	0.99	0.88–1.13	0.93	
Gene	Test	SNP	Position	Alleles	MAF	OR	95% CI	P	
<i>PRKAB2</i>	32	rs750467	143814574	C/T	0.21	0.96	0.85–1.10	0.58	
	33	rs2883434	143873900	A/C	0.26	1.11	0.99–1.25	0.07	
	34	rs1816802	143874874	T/C	0.11	0.97	0.85–1.11	0.70	
	35	rs894467	143886834	T/C	0.05	0.93	0.71–1.21	0.59	
	36	rs1890039	143906767	C/T	0.11	0.86	0.68–1.08	0.19	
			Multi-marker tests		Hap	Freq	OR	95% CI	P
	37		rs750467, rs2883434		C, A	0.55	1.09	0.98–1.20	0.12
	38		rs750467, rs2883434		C, C	0.24	0.88	0.79–0.99	0.04
	39		rs750467, rs1890039		T, C	0.15	1.08	0.93–1.24	0.32
	40		rs2883434, rs1816802		A, T	0.62	1.09	0.99–1.21	0.09

Twenty two single-marker tests and 18 multi-marker haplotype tests were defined based on 22 tag SNPs (9 and 6 for *PRKAA2*, 8 and 8 for *PRKAB1*, and 5 and 4 for *PRKAB2*) and tested for association with type 2 diabetes in our samples. Results from the various samples were combined by Mantel-Haenszel meta-analysis of the ORs. All P values are two-tailed. Chromosomal position is according to the NCBI build 35 release; alleles and OR of individual SNPs are reported as major vs. minor allele.

risk of type 2 diabetes may be due to rare variants not detected by our LD-based methods. We deliberately evaluated SNPs with MAF >5% based on our power calculations, which were designed to detect variants that confer a modest effect on risk in a sample of the size and charac-

teristics available to us. While we believe our tagging methodology to be adequate to capture all common genetic variation (MAF >5%) and our sample size to provide enough power for genotypic relative risks of 1.2 and above, rarer variants or those that have more modest

TABLE 3
Association of variants in *PRKAA2*, *PRKAB1*, and *PRKAB2* with BMI

<i>PRKAA2</i>		BMI (kg/m ²)			
Test	Single-marker	M/M	M/m	mm	<i>P</i>
1	rs2404987	26.8 ± 4.3	26.2 ± 4.9	26.4 ± 4.4	0.26
2	rs6588640	26.4 ± 4.6	26.3 ± 5.1	24.4 ± 2.8	0.21
3	rs2746343	26.3 ± 4.5	26.6 ± 4.6	26.4 ± 6.4	0.18
4	rs857156	26.2 ± 5.0	26.2 ± 4.6	26.7 ± 4.5	0.25
5	rs1342382	26.3 ± 4.4	26.4 ± 7.0	33.3 ± 7.4	0.004*
6	rs3738567	26.2 ± 4.5	26.4 ± 4.9	26.2 ± 5.0	0.56
7	rs857142	26.4 ± 4.1	26.3 ± 5.1	26.4 ± 4.8	0.60
8	rs10489617	26.4 ± 4.7	26.3 ± 4.4	23.7 ± 3.8	0.14
9	rs2275732	26.3 ± 4.7	26.6 ± 4.2	24.5 ± 1.0	0.58
	Multi-marker	11	12	22	
10	2, 3	24.4 ± 2.8	26.9 ± 3.8	26.6 ± 3.7	0.16
11	1, 2, 3	26.5 ± 3.7	26.5 ± 3.6	27.1 ± 3.9	0.07
12	1, 3, 6	25.2 ± 2.8	27.0 ± 3.4	26.6 ± 3.8	0.07
13	3, 4, 5	27.1 ± 4.0	26.6 ± 3.6	26.6 ± 3.7	0.27
14	4, 5	26.5 ± 3.6	26.5 ± 3.6	27.1 ± 4.0	0.04
15	7, 8	26.5 ± 3.5	26.8 ± 3.9	26.4 ± 3.5	0.35
	Multi-marker	11	12	22	
16	rs2015795	26.2 ± 3.5	26.8 ± 3.8	26.9 ± 4.0	0.02
17	rs1541345	26.7 ± 3.7	26.4 ± 4.0	28.0 ± 3.2	0.48
18	rs2393550	26.7 ± 3.8	26.7 ± 3.7	26.1 ± 3.3	0.50
19	rs278143	26.8 ± 3.9	26.5 ± 3.5	26.6 ± 3.7	0.58
20	rs278123	26.8 ± 3.9	26.4 ± 3.4	26.5 ± 3.8	0.25
21	rs278124	26.7 ± 3.7	26.6 ± 3.7	25.7 ± 2.9	0.33
22	rs2285595	27.0 ± 4.1	26.5 ± 3.6	26.5 ± 3.6	0.15
23	rs278109	26.8 ± 3.8	26.4 ± 3.6	26.5 ± 3.4	0.43
	Multi-marker	11	12	22	
24	16, 20	26.1 ± 3.5	26.7 ± 3.7	26.7 ± 3.7	0.26
25	16, 17, 20	26.9 ± 3.9	26.9 ± 3.7	26.2 ± 3.5	0.01
26	16, 19	26.1 ± 3.4	26.6 ± 3.8	26.8 ± 3.7	0.21
27	18, 21	26.0 ± 3.3	26.5 ± 3.6	26.7 ± 3.8	0.42
28	21, 23	26.0 ± 3.4	26.5 ± 3.6	26.8 ± 3.7	0.34
29	18, 20	26.5 ± 3.9	26.4 ± 3.4	26.8 ± 3.9	0.22
30	20, 22, 23	27.0 ± 4.1	26.3 ± 3.4	26.8 ± 3.8	0.09
31	22, 23	27.1 ± 4.1	26.3 ± 3.4	26.8 ± 3.8	0.04
	Multi-marker	11	12	22	
32	rs750467	26.6 ± 3.6	26.6 ± 4.0	27.1 ± 3.4	0.67
33	rs2883434	26.5 ± 3.7	26.7 ± 3.8	27.0 ± 3.7	0.31
34	rs1816802	26.6 ± 3.7	26.7 ± 3.9	26.9 ± 3.4	0.90
35	rs894467	26.7 ± 3.7	26.1 ± 3.6	25.8 ± 0.9	0.43
36	rs1890039	26.7 ± 3.7	26.5 ± 3.8	25.4 ± 1.5	0.78
	Multi-marker	11	12	22	
37	32, 33	26.5 ± 3.6	26.7 ± 3.7	26.9 ± 3.8	0.44
38	32, 33	27.0 ± 3.7	26.7 ± 3.7	26.6 ± 3.7	0.51
39	32, 36	27.0 ± 3.5	26.3 ± 4.0	26.7 ± 3.6	0.26
40	33, 34	26.5 ± 3.6	26.7 ± 3.7	26.9 ± 3.8	0.54

BMI (kg/m²) was determined in our control subjects and logarithmically transformed; untransformed values are presented as mean ± SD. Log-transformed values were compared by ANOVA depending on each of the specified genotypic tests. The SNPs that define each multi-marker test (bottom panel for each gene) are numbered as in the top panel and correspond to those in Table 2. M/M, homozygotes for the major allele; M/m, heterozygotes; m/m, homozygotes for the minor allele. 1 1, two copies of the multi-marker haplotype; 1 2, one copy of the multi-marker haplotype; 2 2, zero copies of the multi-marker haplotype. *The best nominally significant *P* value (*P* = 0.004 for rs1342382) was not statistically significant after 1,000 permutations (*P* = 0.107).

influence on risk may have been missed. In addition, because our tagging tests were selected in the HapMap CEU panel and tested in Caucasian samples, we may have missed variants that are specific to non-European popula-

tions. Nevertheless, we can conclude that the effect of common variants in the AMPK genes *PRKAA2*, *PRKAB1*, and *PRKAB2* on type 2 diabetes, if present, appears to be negligible in Caucasians.

Fourth, overmatching in our samples may have prevented us from detecting a genetic effect of these three AMPK genes on type 2 diabetes if this was mediated through BMI. We explored this possibility by evaluating the effect of the same variants on BMI in our control subjects. None of the tests seemed to have a statistically significant effect on BMI in our sample; this suggests that the impact of common genetic variation in the AMPK genes *PRKAA2*, *PRKAB1*, and *PRKAB2* on BMI is small or absent and may therefore require larger sample sizes for detection.

And finally, common genetic variants in other AMPK genes may still be associated with type 2 diabetes. Given our current negative results with *PRKAA2*, *PRKAB1*, and *PRKAB2*, we have begun characterizing common genetic variation in the remaining four genes for future association studies, although the available haplotype structure from one of the four genes (*PRKAG2*) indicates that this particular gene may not be efficiently evaluated with current LD approaches. In addition, several planned large whole-genome association scans in conjunction with the phase II release of the HapMap (49) may significantly alter the prioritization of individual candidate gene studies.

ACKNOWLEDGMENTS

This work was supported, in part, by a Pilot and Feasibility Grant Award from the Boston Area Diabetes Endocrinology Research Center (BADERC) to J.C.F. and by European Community project EXGENESIS 005272 to L.G. T.T. is a Research Fellow at the Academy of Finland. D.A. is a Charles E. Culpeper Scholar of the Rockefeller Brothers Fund and a Burroughs Wellcome Fund Clinical Scholar in Translational Research. D.A., M.J.D., and J.N.H. are recipients of The Richard and Susan Smith Family Foundation/ADA Pinnacle Program Project Award. L.G., T.T., and the Botnia Study are principally supported by the Sigrid Juselius Foundation, the Academy of Finland, the Finnish Diabetes Research Foundation, The Folkhalsan Research Foundation, the European Community (BM4-CT95-0662, GIFT), the Swedish Medical Research Council, the Juvenile Diabetes Foundation Wallenberg Foundation, and the Novo Nordisk Foundation. J.C.F. is supported by National Institutes of Health Research Career Award 1 K23 DK65978-02.

We thank the members of the Altshuler, Hirschhorn, Daly, and Groop labs for helpful discussions.

NOTE ADDED IN PROOF

The AMPK variants that showed nominal associations with BMI in our Scandinavian samples were genotyped in an additional obese/lean case/control sample totalling 2,873 Caucasian individuals from the U.S. and Poland, provided by Genomics Collaborative. We were unable to replicate any association with BMI, lending further support to the notion that the nominally significant BMI results reported here are due to statistical fluctuation (H.N. Lyon, T. Bersaglieri, K.G. Ardlie, J.N.H., unpublished observations).

REFERENCES

- Florez JC, Hirschhorn JN, Altshuler D: The inherited basis of diabetes mellitus: implications for the genetic analysis of complex traits. *Annu Rev Genomics Hum Genet* 4:257–291, 2003
- O’Rahilly S, Barroso I, Wareham NJ: Genetic factors in type 2 diabetes: the end of the beginning? *Science* 307:370–373, 2005
- Hardie DG: The AMP-activated protein kinase cascade: the key sensor of cellular energy status. *Endocrinology* 144:5179–5183, 2003
- Zong H, Ren JM, Young LH, Pypaert M, Mu J, Birnbaum MJ, Shulman GI: AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation. *Proc Natl Acad Sci U S A* 99:15983–15987, 2002
- Fisher JS, Gao J, Han DH, Holloszy JO, Nolte LA: Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. *Am J Physiol Endocrinol Metab* 282:E18–E23, 2002
- Viollet B, Andreelli F, Jorgensen SB, Perrin C, Geloan A, Flamez D, Mu J, Lenzner C, Baud O, Bennoun M, Gomas E, Nicolas G, Wojtaszewski JF, Kahn A, Carling D, Schuit FC, Birnbaum MJ, Richter EA, Burcelin R, Vaulont S: The AMP-activated protein kinase $\alpha 2$ catalytic subunit controls whole-body insulin sensitivity. *J Clin Invest* 111:91–98, 2003
- Minokoshi Y, Kim YB, Peroni OD, Fryer LG, Muller C, Carling D, Kahn BB: Leptin stimulates fatty-acid oxidation by activating AMP-activated protein kinase. *Nature* 415:339–343, 2002
- Hayashi T, Hirshman M, Fujii N, Habinowski S, Witters L, Goodyear L: Metabolic stress and altered glucose transport: activation of AMP-activated protein kinase as a unifying coupling mechanism. *Diabetes* 49:527–531, 2000
- Fujii N, Hayashi T, Hirshman MF, Smith JT, Habinowski SA, Kaijser L, Mu J, Ljungqvist O, Birnbaum MJ, Witters LA: Exercise induces isoform-specific increase in 5' AMP-activated protein kinase activity in human skeletal muscle. *Biochem Biophys Res Commun* 273:1150–1155, 2000
- Wojtaszewski JF, Nielsen P, Hansen BF, Richter EA, Kiens B: Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle. *J Physiol* 528:221–226, 2000
- Stephens TJ, Chen ZP, Canny BJ, Michell BJ, Kemp BE, McConell GK: Progressive increase in human skeletal muscle AMPK $\alpha 2$ activity and ACC phosphorylation during exercise. *Am J Physiol Endocrinol Metab* 282: E688–E694, 2002
- Musi N, Fujii N, Hirshman MF, Ekberg I, Froberg S, Ljungqvist O, Thorell A, Goodyear LJ: AMP-activated protein kinase (AMPK) is activated in muscle of subjects with type 2 diabetes during exercise. *Diabetes* 50:921–927, 2001
- Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, Zhou G, Williamson JM, Ljunqvist O, Efendic S, Moller DE, Thorell A, Goodyear LJ: Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes* 51:2074–2081, 2002
- Zang M, Zuccollo A, Hou X, Nagata D, Walsh K, Herscovitz H, Brecher P, Ruderman NB, Cohen RA: AMP-activated protein kinase is required for the lipid-lowering effect of metformin in insulin-resistant human HepG2 cells. *J Biol Chem* 279:47898–47905, 2004
- Fryer LGD, Parbu-Patel A, Carling D: The anti-diabetic drugs rosiglitazone and metformin stimulate AMP-activated protein kinase through distinct signaling pathways. *J Biol Chem* 277:25226–25232, 2002
- Leclerc I, Woltersdorf WW, da Silva Xavier G, Rowe RL, Cross SE, Korbitt GS, Rajotte RV, Smith R, Rutter GA: Metformin, but not leptin, regulates AMP-activated protein kinase in pancreatic islets: impact on glucose-stimulated insulin secretion. *Am J Physiol Endocrinol Metab* 286:E1023–E1031, 2004
- Hawley SA, Gadalla AE, Olsen GS, Hardie DG: The antidiabetic drug metformin activates the AMP-activated protein kinase cascade via an adenine nucleotide-independent mechanism. *Diabetes* 51:2420–2425, 2002
- Sakamoto K, Goransson O, Hardie DG, Alessi DR: Activity of LKB1 and AMPK-related kinases in skeletal muscle: effects of contraction, phenformin, and AICAR. *Am J Physiol Endocrinol Metab* 287:E310–E317, 2004
- Mori Y, Otabe S, Dina C, Yasuda K, Populaire C, Lecoecur C, Vatin V, Durand E, Hara K, Okada T, Tobe K, Boutin P, Kadowaki T, Froguel P: Genome-wide search for type 2 diabetes in Japanese affected sib-pairs confirms susceptibility genes on 3q, 15q, and 20q and identifies two new candidate loci on 7p and 11p. *Diabetes* 51:1247–1255, 2002
- Viollet B, Andreelli F, Jorgensen SB, Perrin C, Flamez D, Mu J, Wojtaszewski JF, Schuit FC, Birnbaum M, Richter E, Burcelin R, Vaulont S: Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models. *Biochem Soc Trans* 31:216–219, 2003
- Musi N, Yu H, Goodyear LJ: AMP-activated protein kinase regulation and action in skeletal muscle during exercise. *Biochem Soc Trans* 31:191–195, 2003
- Mahtani MM, Widen E, Lehto M, Thomas J, McCarthy M, Brayer J, Bryant B, Chan G, Daly M, Forsblom C, Kanninen T, Kirby A, Kruglyak L, Munnally K, Parkkonen M, Reeve-Daly MP, Weaver A, Brettin T, Duyk G, Lander ES, Groop LC: Mapping of a gene for type 2 diabetes associated with an insulin secretion defect by a genome scan in Finnish families. *Nat Genet* 14:90–94, 1996
- Shaw JT, Lovelock PK, Kesting JB, Cardinal J, Duffy D, Wainwright B,

- Cameron DP: Novel susceptibility gene for late-onset NIDDM is localized to human chromosome 12q. *Diabetes* 47:1793–1796, 1998
24. Bowden DW, Sale M, Howard TD, Qadri A, Spray BJ, Rothschild CB, Akots G, Rich SS, Freedman BI: Linkage of genetic markers on human chromosomes 20 and 12 to NIDDM in Caucasian sib pairs with a history of diabetic nephropathy. *Diabetes* 46:882–886, 1997
 25. Warden SM, Richardson C, O'Donnell J Jr., Stapleton D, Kemp BE, Witters LA: Post-translational modifications of the β -1 subunit of AMP-activated protein kinase affect enzyme activity and cellular localization. *Biochem J* 354:275–283, 2001
 26. Polekhina G, Gupta A, Michell BJ, van Denderen B, Murthy S, Feil SC, Jennings IG, Campbell DJ, Witters LA, Parker MW: AMPK β subunit targets metabolic stress sensing to glycogen. *Current Biology* 13:867–871, 2003
 27. Sakoda H, Fujishiro M, Fujio J, Shojima N, Ogihara T, Kushiya A, Fukushima Y, Anai M, Ono H, Kikuchi M, Horike N, Viana AYI, Uchijima Y, Kurihara H, Asano T: Glycogen debranching enzyme association with β -subunit regulates AMP-activated protein kinase activity. *Am J Physiol Endocrinol Metab* 289:E474–E481, 2005
 28. Hanson RL, Ehm MG, Pettitt DJ, Prochazka M, Thompson DB, Timberlake D, Foroud T, Kobes S, Baier L, Burns DK, Almasy L, Blangero J, Garvey WT, Bennett PH, Knowler WC: An autosomal genomic scan for loci linked to type II diabetes mellitus and body-mass index in Pima Indians. *Am J Hum Genet* 63:1130–1138, 1998
 29. Elbein SC, Hoffman MD, Teng K, Leppert MF, Hasstedt SJ: A genome-wide search for type 2 diabetes susceptibility genes in Utah Caucasians. *Diabetes* 48:1175–1182, 1999
 30. Ng MCY, So W-Y, Lam VKL, Cockram CS, Bell GI, Cox NJ, Chan JCN: Genome-wide scan for metabolic syndrome and related quantitative traits in Hong Kong Chinese and confirmation of a susceptibility locus on chromosome 1q21–q25. *Diabetes* 53:2676–2683, 2004
 31. Vionnet N, Hani El H, Dupont S, Gallina S, Francke S, Dotte S, De Matos F, Durand E, Lepretre F, Lecoeur C, Gallina P, Zekiri L, Dina C, Froguel P: Genomewide search for type 2 diabetes-susceptibility genes in French whites: evidence for a novel susceptibility locus for early-onset diabetes on chromosome 3q27-qter and independent replication of a type 2-diabetes locus on chromosome 1q21–q24. *Am J Hum Genet* 67:1470–1480, 2000
 32. Thornton C, Snowden MA, Carling D: Identification of a novel AMP-activated protein kinase β subunit isoform that is highly expressed in skeletal muscle. *J Biol Chem* 273:12443–12450, 1998
 33. Wojtaszewski JFP, Birk JB, Frogis C, Holten M, Pilegaard H, Dela F: 5' AMP activated protein kinase expression in human skeletal muscle: effects of strength training and type 2 diabetes. *J Physiol (Lond)* 564:563–573, 2005
 34. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, Zhang J, Soden R, Hayakawa M, Kreiman G, Cooke MP, Walker JR, Hogenesch JB: A gene atlas of the mouse and human protein-encoding transcriptomes. *Proc Natl Acad Sci U S A* 101:6062–6067, 2004
 35. Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D: The structure of haplotype blocks in the human genome. *Science* 296:2225–2229, 2002
 36. Tang K, Fu DJ, Julien D, Braun A, Cantor CR, Koster H: Chip-based genotyping by mass spectrometry. *Proc Natl Acad Sci U S A* 96:10016–10020, 1999
 37. de Bakker PIW, Yelensky R, Pe'er I, Gabriel SB, Daly MJ, Altshuler D: Efficiency and power in genetic association studies. *Nat Genet* 37:1217–1223, 2005
 38. Barrett JC, Fry B, Maller J, Daly MJ: Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* 21:263–265, 2005
 39. Florez JC, Agapakis CM, Burt NP, Sun M, Almgren P, Rastam L, Tuomi T, Gaudet D, Hudson TJ, Daly MJ, Ardlie KG, Hirschhorn JN, Groop L, Altshuler D: Association testing of the protein tyrosine phosphatase 1B gene (*PTPN1*) with type 2 diabetes in 7,883 people. *Diabetes* 54:1884–1891, 2005
 40. Altshuler D, Hirschhorn JN, Klannemark M, Lindgren CM, Vohl MC, Nemesh J, Lane CR, Schaffner SF, Bolk S, Brewer C, Tuomi T, Gaudet D, Hudson TJ, Daly M, Groop L, Lander ES: The common PPAR γ Pro12Ala polymorphism is associated with decreased risk of type 2 diabetes. *Nat Genet* 26:76–80, 2000
 41. Winckler W, Graham RR, de Bakker PIW, Sun M, Almgren P, Tuomi T, Gaudet D, Hudson TJ, Ardlie KG, Daly MJ, Hirschhorn JN, Groop L, Altshuler D: Association testing of variants in the hepatocyte nuclear factor 4 α gene with risk of type 2 diabetes in 7,883 people. *Diabetes* 54:886–892, 2005
 42. Florez JC, Burt N, de Bakker PIW, Almgren P, Tuomi T, Holmkvist J, Gaudet D, Hudson TJ, Schaffner SF, Daly MJ, Hirschhorn JN, Groop L, Altshuler D: Haplotype structure and genotype-phenotype correlations of the sulfonylurea receptor and the islet ATP-sensitive potassium channel gene region. *Diabetes* 53:1360–1368, 2004
 43. Purcell S, Cherny SS, Sham PC: Genetic power calculator: design of linkage and association genetic mapping studies of complex traits. *Bioinformatics* 19:149–150, 2003
 44. Spielman RS, McGinnis RE, Ewens WJ: Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 52:506–516, 1993
 45. Boehnke M, Langefeld CD: Genetic association mapping based on discordant sib pairs: the discordant-alleles test. *Am J Hum Genet* 62:950–961, 1998
 46. Lohmueller K, Pearce CL, Pike M, Lander ES, Hirschhorn JN: Meta-analysis of genetic association studies supports a contribution of common variants to susceptibility to common disease. *Nat Genet* 33:177–182, 2003
 47. Matsuda M, DeFronzo R: Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22:1462–1470, 1999
 48. Bergeron R, Russell RR, III, Young LH, Ren J-M, Marcucci M, Lee A, Shulman GI: Effect of AMPK activation on muscle glucose metabolism in conscious rats. *Am J Physiol Endocrinol Metab* 276:E938–E944, 1999
 49. Altshuler D, Brooks LD, Chakravarti A, Collins FS, Daly MJ, Donnelly P, International HapMap Consortium: A haplotype map of the human genome. *Nature* 437:1299–1320, 2005