Original Article

A Functional Tyr1306Cys Variant in LARG Is Associated With Increased Insulin Action in Vivo

Peter Kovacs,[1,2] Michael Stumvoll,[2] Clifton Bogardus,[1] Robert L. Hanson,[1] and Leslie J. Baier[1]

Diminished insulin sensitivity is a characteristic feature of type 2 diabetes. Inhibition of insulin action, resulting in reduced skeletal muscle glucose uptake, is mediated in part through stimulation of RhoA activity. One regulator of RhoA activity is leukemia-associated Rho guanine nucleotide exchange factor (*LARG*). The *LARG* gene maps to a region on chromosome 11q23-24 that shows genetic linkage to BMI and type 2 diabetes in Pima Indians. Because of its role in RhoA activation, the *LARG* gene was analyzed as a positional candidate gene for this linkage. Sequencing of the *LARG* gene and genotyping of variants identified several polymorphisms that were associated with in vivo rates of insulin-mediated glucose uptake, at both physiological and maximally stimulating insulin concentrations, among 322 nondiabetic Pima Indians who had undergone a hyperinsulinemic-euglycemic clamp. The strongest association with rate of glucose uptake was found with a Tyr1306Cys polymorphism ($P < 0.0001$, adjusted for age, sex, percent body fat, and nuclear family membership). In transient transfection studies in NIH3T3 cells, the LARG(Cys1306) protein had reduced activity compared with LARG(Tyr1306) protein ($P < 0.05$). We propose that the Tyr1306Cys substitution in LARG, through its differential activation of RhoA, increases insulin sensitivity in nondiabetic Pima Indians. *Diabetes* 55:1497–1503, 2006

The prevalence of type 2 diabetes has reached epidemic proportions in many developed countries. The number of people worldwide with diabetes is currently >150 million, and this number is projected to double by the year 2025 (1). The escalating rate of type 2 diabetes is likely due to changes in the environment, coupled with changes in human behavior and lifestyle. However, a genetic component, independent of environment, has also been demonstrated in classical twin-, family-, and population-based studies (2). The genetic component of type 2 diabetes appears to be polygenic and complex in nature. Several groups have sought to reduce the "complexity" of type 2 diabetes by studying this disease within populations that have limited genetic and environmental variability. The Pima Indians of the Gila River Indian Community is one population that has been studied extensively. Members of this Native American community have participated in longitudinal studies of the etiology of diabetes since 1965 and have the highest reported prevalence of type 2 diabetes of any population in the world (3). Their diabetes is prototypic of type 2 diabetes in that it is metabolically characterized by obesity, insulin resistance, insulin secretory dysfunction, and increased rates of endogenous glucose production, and a subset of this population has participated in detailed in vivo physiological studies to quantify these metabolic characteristics. In prospective studies of pre-diabetic Pima Indians, insulin resistance and insulin secretory dysfunction, independent of obesity, are major predictors of the disease (4). Insulin resistance results in part from obesity, but some determinants of impaired insulin action are independent of fatness.

To search for genetic loci that contribute to the high prevalence of type 2 diabetes and obesity among the Pima Indians, we completed a genomic scan in >1,200 Pima Indians from 264 nuclear families (5). Variance-components methods were used to test for linkage with an age-adjusted diabetes score and with BMI. In multipoint analyses, the strongest evidence for linkage with BMI (logarithm of odds [LOD] = 3.6) was centered at marker D11S4464 on chromosome 11q. This region also showed suggestive linkage to age-adjusted diabetes (LOD = 1.7). Bivariate linkage analysis for the combined phenotype "diabesity" gave the strongest evidence for linkage (*P* value equivalent to LOD = 5.0), suggesting that this putative locus influences obesity and young-onset diabetes. The region of linkage is positioned at 11q23-24 and spans ~24 Mb.

To identify the genetic variant(s) that gave rise to the linkage to type 2 diabetes and BMI in the Pima Indians, positional candidate genes that map near 11q23-24 are being analyzed. One such candidate is the leukemia-associated Rho guanine nucleotide exchange factor (*LARG*) gene. This gene was originally identified by Kourlas et al. (6) and contains strong sequence homology to several members of the Rho family of guanine nucleotide exchange factors (GEFs). Rho is active when bound to GTP and inactive when bound to GDP, and this cycling between active and inactive Rho is regulated by RhoGEFs that stimulate the exchange of GDP for GTP. Recent studies have shown that LARG is involved in the activation pathway of RhoA, and stimulation of RhoA activity results

From the [1]Phoenix Epidemiology and Clinical Research Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona; and the [2]Medical Department III, University of Leipzig, Leipzig, Germany.

Address correspondence and reprint requests to Leslie J. Baier, PhD, Diabetes Molecular Genetics Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, 445 North 5th St., Phoenix, AZ 85004. E-mail: lbaier@phx.niddk.nih.gov.

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GEF, guanine nucleotide exchange factor; LARG, leukemia-associated Rho guanine nucleotide exchange factor; LD, linkage disequilibrium; LPA, lysophosphatidic acid; PDZ, postsynaptic density disc-large zo-1; SNP, single nucleotide polymorphism; SRE, serum response element; TCF, ternary complex factor; UTR, untranslated region.

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TABLE 1
Main characteristics of genetic variants detected in the *LARG* and its 5' region

Variant	Position within the gene (nucleotide)	Alleles	Minor allele frequency	GenBank position	DbSNP (BUILD 124)
LARG-SNP1	5' UTR (−8029)	T/C	C = 0.33	98864 (AP001150.4)	—
LARG-SNP2	5' UTR (−5854)	T/C	C = 0.33	101039 (AP001150.4)	rs11217821
LARG-SNP3	5' UTR (−5659)	G/A	A = 0.42	101234 (AP001150.4)	—
LARG-SNP4	5' UTR (−4695)	T/C	C = 0.12	102198 (AP001150.4)	—
LARG-SNP5	5' UTR (−4394)	A/G	G = 0.33	102499 (AP001150.4)	—
LARG-SNP6	5' UTR (−4325)	G/A	A = 0.33	102568 (AP001150.4)	rs12806740
LARG-SNP7	5' UTR (−2343)	TA (repeat)	TA (del) = 0.39	104498 (AP001150.4)	—
LARG-SNP8	5' UTR (−317)	G (ins/del)	G (del) = 0.28	105576 (AP001150.4)	rs3840762
LARG-SNP9	5' UTR (−548)	A/G	G = 0.33	106345 (AP001150.4)	rs7126413
LARG-SNP10	Intron 2 (−88)	T/C	C = 0.28	66021 (AP000681.3)	rs6589812
LARG-SNP11	Intron 12 (199)	A/G	G = 0.28	129684 (AC016034.2)	rs10892578
LARG-SNP12	Intron 12 (−987)	C/T	T = 0.12	131245 (AC016034.2)	rs538661
LARG-SNP13	Intron 14 (−67)	G/A	A = 0.28	134140 (AC016034.2)	rs723937
LARG-SNP14	Intron 20 (7)	A/C	C = 0.28	140461 (AC016034.2)	rs2305008
LARG-SNP15	Intron 22 (−1549)	G/A	A = 0.39	147675 (AC016034.2)	rs476636
LARG-SNP16	Intron 29 (23)	A/G	G = 0.39	159437 (AC016034.2)	rs2305011
LARG-SNP17	Intron 34 (−43)	GTTGT (ins/del)	GTTGT (ins) = 0.28	9563 (AP000681.3)	—
LARG-SNP18	Exon 38 (Tyr1306Cys)	A/G (T<u>A</u>T/T<u>G</u>T)*	G = 0.42	3924 (NM_015313)	—
LARG-SNP19	Intron 40 (14)	A/G	G = 0.12	71241 (AP000681.3)	rs503473
LARG-SNP20	3'UTR (6287)	T (ins/del)	T (del) = 0.28	6287 (NM_015313)	rs3832734
LARG-SNP21	3'UTR (6699)	TT (ins/del)	TT (del) = 0.28	6699 (NM_015313)	—

* Underlined letters indicate the position of ATG substitution within codon 1306.

in reduced skeletal muscle glucose transport (7–9). Therefore, *LARG*, whose protein product can activate Rho, was investigated as a positional candidate gene for type 2 diabetes and related phenotypes.

RESEARCH DESIGN AND METHODS

All subjects (n = 1,346) are participants of the ongoing longitudinal studies of the etiology of type 2 diabetes among the Gila River Indian Community in Arizona (3). Diabetes status for these subjects was determined by an oral glucose tolerance test and interpreted according to the criteria of the World Health Organization (10). A subset of the nondiabetic subjects had also been admitted as inpatients to our clinical research center for detailed metabolic testing. Oral glucose tolerance was determined after an overnight fast. Subjects ingested 75 g glucose, and blood for plasma glucose and insulin measurements was drawn before ingesting the glucose and at 30, 60, 120, and 180 min thereafter. On a different day, subjects also received a 25-g intravenous injection of glucose over 3 min to measure the acute insulin response, as described previously (11). Blood samples were collected before infusion and at 3, 4, 5, 6, 8, and 10 min after infusion for determination of plasma glucose and insulin concentrations. The acute insulin response was calculated as one-half the mean increment in plasma insulin concentrations from 3 to 5 min.

The hyperinsulinemic-euglycemic clamp technique was used to determine basal glucose appearance and insulin-stimulated glucose disappearance (uptake) rates, as described in detail previously (4,11). Briefly, insulin was infused to achieve physiological and maximally stimulating plasma insulin concentrations (137 ± 3 and 2,394 ± 68 μU/ml, respectively) for 100 min for each step. Plasma glucose concentrations were held constant at ~100 mg/dl by a variable 20% glucose infusion. Tritiated glucose was infused beginning 2 h before the insulin infusion and continued until completion of the low-dose infusion to calculate rates of postabsorptive glucose appearance and glucose disappearance during the lower dose of insulin infusion (4).

Body composition was estimated by underwater weighing until January 1996 and by dual-energy X-ray absorptiometry (DPX-1; Lunar Radiation, Madison, WI) thereafter. A conversion equation derived from comparative analyses was used to make estimates of body composition equivalent between methods (12).

All studies were approved by the Tribal Council and the Institutional Review Board of the National Institutes of Diabetes and Digestive and Kidney Diseases.

Sequencing of the *LARG* gene. Genomic DNA from 10 nondiabetic insulin-sensitive and 10 nondiabetic insulin-resistant Pima Indians was selected for variant detection. None of these individuals were first-degree relatives among themselves. All 41 exons, including intron/exon splicing sites, the 5′- and 3′-untranslated regions (UTRs), and 8 kb of the upstream (putative promoter) region of the *LARG* gene, were sequenced in the 20 DNA samples. Sequencing was performed using the Big Dye Terminator on an automated DNA sequencer (models 377 and 3700; Applied Biosystems). Sequence information of oligonucleotides is available upon request.

Genotyping of single nucleotide polymorphisms. Genotyping of selected single nucleotide polymorphisms (SNPs) in DNA from 1,346 Pima Indians was done by either the TaqMan assay (for SNPs LARG-SNP6, LARG-SNP11, LARG-SNP12, and LARG-SNP18) or Pyrosequencing (for LARG-SNP15). The TaqMan genotyping reaction was run on a GeneAmp PCR system 9700 (50°C for 2 min, 95°C for 10 min, and 95°C for 15 s, and 62°C for 1 min for 38 cycles), and fluorescence was detected on an ABI Prism 7700 sequence detector (Applied Biosystems). The Pyrosequencing reaction (LARG-SNP15) was run on GeneAmp PCR System 9700 (95°C for 10 min, 95°C for 30 s, 55°C for 1 min, 72°C for 1 min for 38 cycles, and 72°C for 10 min) and was analyzed on PSQ96 sequencer (Pyrosequencing, Uppsala, Sweden). Genotypes of the 1,346 subjects were analyzed for association with type 2 diabetes and BMI. In addition, the genotypes from a subset (n = 322) of the nondiabetic subjects who had undergone additional metabolic studies were analyzed for associations with percent body fat, 2-h plasma insulin, 2-h plasma glucose, and glucose disposal at both physiological and maximally stimulating plasma insulin concentrations.

Quantitation of *LARG* expression in human skeletal muscle. Messenger RNA was extracted from skeletal muscle biopsies of 17 nondiabetic, insulin-resistant Pima Indians and 18 insulin-sensitive Pima Indians. Subjects were matched for age, sex, and percent body fat. Percutaneous muscle biopsies were obtained from the quadriceps femoris muscle after local anesthesia of skin and fascia with 2% lidocaine. Biopsies were immediately frozen in liquid nitrogen, and RNA was extracted using a ToTALLY RNA kit (Ambion, Austin, TX). Quantitation of *LARG* expression was performed using TaqMan real-time PCR on cDNA synthesized from total RNA. The primer pairs and probe are available upon request. The quantification was performed using the standard protocol of ABI PRISM 7700 (Applied Biosystems). Each sample was run in duplicate, and the mean value of the duplicate was used to calculate transcript level. The transcript quantity in each cDNA sample was normalized to that of cyclophilin using the TaqMan Pre-Developed Assay Reagent for human endogenous controls (Applied Biosystems).

In vitro functional assay of Tyr1306Cys. LARG is known to activate Rho, which in turn induces expression from serum response elements (SREs) through the activation of the serum response factor (13,14).

Because studies using the c-*fos* promoter demonstrate that Rho can activate transcription through the SRE independent of effects of ternary complex factor (TCF) (15), we used this system as a readout for LARG-mediated activation of Rho and consequently of the SRE.L-Luc, lacking the

TABLE 2
LARG variants grouped by LD ($r^2 = 1$) among 20 Pima Indians

Group 1	Group 2	Group 3	Group 4	Group 5
LARG-SNP8	LARG-SNP1	LARG-SNP7	LARG-SNP4	LARG-SNP3
LARG-SNP10	LARG-SNP2	**LARG-SNP15**	**LARG-SNP12**	**LARG-SNP18**
LARG-SNP11	**LARG-SNP6**	LARG-SNP16	LARG-SNP19	
LARG-SNP13	LARG-SNP5			
LARG-SNP14	LARG-SNP9			
LARG-SNP17				
LARG-SNP20				
LARG-SNP21				

Representative SNPs for additional genotyping are in bold.

TCF binding site. Transient transfection experiments were used to determine whether the Tyr1306Cys could affect the ability of LARG to activate Rho and thereby alter levels of serum response factors. An expression plasmid LARG(WT)-pCGN, which encodes the LARG protein, was provided by Dr. G.W. Reuther (University of North Carolina) (16). Site-directed mutagenesis (QuickChange XL; Stratagene) was used to introduce an A-to-G mutation at the second position of codon 1,306 in the *LARG* gene, and the mutant plasmid was designated as LARG(M)-pCGN. A reporter plasmid encoding the luciferase gene, under the control of mutant SRE from the *c-fos* promoter that lacks the TCF-binding site (SRE.L-Luc), was provided by Dr. K. Kaibuchi (Nagoya University, Nagoya, Japan) (17).

Cell line and transfection. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Expression plasmids LARG(WT)-pCGN and LARG(M)-pCGN were cotransfected into NIH3T3 cells with the luciferase reporter plasmid SRE.L-Luc plasmid using LipofectAMINE Plus according to manufacturer's protocol. A pRL-TK (*Renilla* luciferase) plasmid was used as an internal control serving as the baseline response (Dual-Luciferase Reporter 1000 Assay; Promega). At 24 h after transfection, cells were placed in Dulbecco's modified Eagle's medium supplemented with 0.5% fetal bovine serum and incubated for an additional 20 h. Cell lysates were then analyzed for luciferase (firefly and *Renilla*) activities using a LUMI-SCINT luminometer (Bioscan, Washington, DC). Transfections were carried out in six-well plates in duplicate, and the data shown are average of six experiments (±SE).

Statistics. Statistical evaluations of the data were performed using the Statistical Analysis System of the SAS Institute (Cary, NC). The generalized estimating equation procedure was used to adjust for the covariates; these generalized estimating equation models were fit taking family membership into account, because some subjects were siblings. Plasma insulin concentration and rates of glucose disappearance during the physiological dose insulin infusion were log transformed before analyses to approximate a normal distribution.

RESULTS

Genetic variants in *LARG*. Twenty-one variants in *LARG* were identified by sequencing 20 subjects (Table 1). Only one of these variants was positioned within a coding region. This A/G substitution (LARG-SNP18) in exon 38 predicts a tyrosine to cysteine substitution at codon 1,306 (Tyr1306Cys). Nine other variants (eight SNPs and one insertion) were positioned within intronic sequences, and two variants (both deletions) were detected in the 3′-UTR region. In addition, nine variants were positioned within the upstream UTR. Based on the genotypic data from 20 Pima Indians that were sequenced, five linkage disequilibrium (LD) groups could be established for these 21 variants (Table 2). SNPs within each LD group were in complete LD ($r^2 = 1$).

Association studies. One representative SNP from each LD group was genotyped for association analyses in 1,346 ($n = 810$ diabetic and $n = 536$ nondiabetic) Pima Indian subjects.

LARG-SNP6, LARG-SNP11, LARG-SNP12, LARG-SNP15, and LARG-SNP18 were selected as representatives for each of the five LD groups (Table 2). The genotype distribution of all SNPs was consistent with Hardy-Weinberg equilibrium. Results of the Hardy-Weinberg equilibrium test were as follows: LARG-SNP6 ($P = 0.54$), LARG-SNP11 ($P = 0.69$), LARG-SNP12 ($P = 0.18$), LARG-SNP15 ($P = 0.32$), and LARG-SNP18 ($P = 0.12$). Based on the genotypic data from the 1,346 subjects, D′ and r^2 were calculated between each of the representative SNPs (Table 3). D′ was very high (>0.98) between all pairs of SNPs except for LARG SNP12 and LARG SNP6 (D′ = 0.22). None of the SNPs was associated with type 2 diabetes or BMI among the 1,346 subjects (data not shown). We estimated the power of the sample to be >74% to detect an association with a functional variant (of frequency >0.05) that accounts for >1% of the variance in liability to diabetes (18).

However, among 322 of the nondiabetic subjects who had additionally undergone detailed metabolic phenotyping that included a hyperinsulinemic-euglycemic clamp, LARG-SNP6, LARG-SNP11, and LARG-SNP15 (representing LD groups 2, 1, and 3, respectively) were associated with insulin action at maximally stimulating plasma insulin concentrations ($P < 0.05$, after adjusting for age, sex, and percent body fat) (Table 4). In addition, the Tyr1306Cys (LARG-SNP18) variation was highly associated with insulin action at both physiological ($P = 0.0001$) and maximally stimulating ($P = 0.004$) plasma insulin concentrations (Table 4).

In addition, among the five genotyped SNPs, there were five common haplotypes (frequency >0.05) that accounted for 99.7% of the estimated haplotypes. These common haplotypes could be defined by four of the SNPs ([SNP6]-[SNP11]-[SNP12]-[SNP18]). Each of the common haplotypes was analyzed for its association with diabetes and metabolic variables. This was accomplished by a modification of the zero-recombination haplotyping approach

TABLE 3
LD between representative SNPs from the five LD groups

	LARG SNP6	LARG SNP11	LARG SNP12	LARG SNP15	LARG SNP18
LARG SNP6	1	0.99	0.22	0.98	0.99
LARG SNP11	0.75	1	0.99	0.99	0.99
LARG SNP12	0.01	0.05	1	0.99	0.99
LARG SNP15	0.72	0.56	0.21	1	0.99
LARG SNP18	0.35	0.27	0.10	0.47	1

D′ given in upper shaded boxes and r^2 given in lower boxes, calculated from genotypic data from 1,346 subjects.

TABLE 4
Association of *LARG* SNPs with diabetes-related phenotypes

	LARG-SNP6				LARG-SNP11			
Genotype	AA (n = 148)	AG (n = 137)	GG (n = 37)	P	AA (n = 165)	AG (n = 133)	GG (n = 24)	P
Male/females (n)	89/59	84/53	18/19	0.37	100/65	82/51	9/15	0.08
Percent body fat	32 ± 1	32 ± 1	33 ± 1	0.85	32 ± 1	32 ± 1	33 ± 2	0.57
2-h plasma glucose (mg/dl)	121 ± 3	126 ± 3	131 ± 5	0.19	121 ± 2	126 ± 2	138 ± 6	0.07
(log)2-h plasma insulin	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	0.60	2.1 ± 0.1	2.2 ± 0.1	2.3 ± 0.1	0.16
(log)glucose disposal for low-dose insulin clamp (mg · kg EMBS^{-1} · min^{-1})	0.41 ± 0.01	0.39 ± 0.01	0.38 ± 0.02	0.22	0.41 ± 0.01	0.40 ± 0.01	0.36 ± 0.02	0.07
Glucose disposal for high-dose insulin clamp (mg · kg EMBS^{-1} · min^{-1})	9.1 ± 0.2	8.7 ± 0.2	8.0 ± 0.3	0.03	9.0 ± 0.2	8.7 ± 0.2	7.6 ± 0.4	0.02

Data are means ± SE. P values were calculated after adjusting for age, sex, nuclear family membership for the variable percent body fat and for age, sex, percent body fat, and nuclear family membership for the variables 2-h plasma glucose (2 h after ingestion of 75 g glucose for oral glucose tolerance test), 2-h plasma insulin, and $\log_{10}$ glucose disposal for the physiologic and maximally stimulating insulin clamps. Due to the low frequency of the rare alleles for LARG-SNP12 variants, for statistical analysis, the homozygotes for each rare allele were combined with the heterozygotes; therefore, only a dominant effect on risk has been tested for the rare allele. EMBS, estimated metabolic body size.

(19–21). Analyses of these haplotypes did not reveal any associations beyond those expected from analysis of individual SNPs.

Functional role of *LARG* variants. To determine whether any of the sequence variants alter in vivo LARG expression, real-time PCR measurements were made on mRNA extracted from skeletal muscle biopsies of 17 nondiabetic, insulin-resistant Pima Indians and 18 insulin-sensitive Pima Indians. No relationship was found between an individual's *LARG* message level and his/her genotype for any of the six SNPs (data not shown).

In addition, in vitro functional studies were performed to determine whether the Tyr1306Cys alters the function of the protein. Repeated 48-h transient transfections of plasmids expressing either Tyr- or Cys-containing LARG protein in NIH3T3 cells showed that cells cotransfected with a reporter SRE.L-Luciferase (luciferase activity expressed under control of a SRE.L) and LARG(Tyr1306) had a 100 ± 11% increase in luciferase expression compared with cells transfected with the reporter alone. In contrast, cells cotransfected with LARG(Cys1306) and the reporter SRE.L-Luciferase increased the expression by 73 ± 10% compared with the reporter alone (Fig. 1). The difference in activation between the LARG(Tyr1306) and LARG-(Cys1306) constructs was statistically significant (P = 0.03).

DISCUSSION

The predicted protein product of the *LARG* gene contains several structural domains (postsynaptic density disc-large zo-1 [PDZ] domain, regulator of G-protein signaling domain, Dbl homology domain, and pleckstrin homology domain) that are conserved among family members of RhoGEFs. LARG can activate RhoA, a member of the Rho family of GTPases (e.g., Cdc42, Rac, and Ras) that are crucial in diverse cellular events, including cell cycle regulation, DNA synthesis, gene transcription, and smooth muscle contraction (22–24). Activation of RhoA appears to occur by a variety of different stimulants. For example, lysophosphatidic acid (LPA) induces a variety of biological responses via heterotrimeric G-protein–coupled receptors, including LPA1 and LPA2. It has recently been shown that the COOH terminus of LPA1 and LPA2 can interact with the PDZ domain of LARG to cause LPA-induced RhoA activation (7). Interaction between the PDZ domain of LARG and Plexin-B1 is also important for Sema4D activation of RhoA (25,26). Reconstitution and transfection studies have demonstrated that LARG also interacts with G-proteins Gα12 and Gα13 via its regulator of G-protein signaling domain, to stimulate RhoA activity (27–32). LARG is also critically involved in Gq/G11-mediated RhoA activation (33).

LARG also has a role in IGF-1–dependent signal transduction pathways and cytoskeletal rearrangements (34). Expression studies have shown that LARG protein colocalizes with the IGF-1 receptor in various tissues, suggesting a physiological role for LARG as an activator of RhoA in response to IGF-1 (8). There is also evidence that the IGF-1 receptor interacts directly with the PDZ domain of LARG, indicating that IGF-1 regulates downstream signal transduction pathways by activating the Rho/Rho-kinase pathway via a LARG/IGF-1 complex (34).

In the present study, we identified a number of variants in the *LARG* gene that are associated with rates of insulin-mediated glucose uptake in vivo, as determined by a hyperinsulinemic-euglycemic clamp study. LARG-SNP18, which predicts a tyrosine to cysteine substitution (Tyr1306Cys), was the most highly associated SNP, whereby nondiabetic subjects homozygous for the Cys allele had a higher mean glucose disposal rate at both physiological and maximally stimulating insulin concentrations compared with nondiabetic subjects homozygous for the Tyr allele (Table 4). In vivo studies comparing expression levels of both of these alleles in muscle indicated that the Cys allele and the Tyr allele had similar levels of expression, but in vitro transient transfection studies showed that the LARG protein with a cysteine at position 1,306 had reduced activity compared with LARG protein with a tyrosine at position 1,306. These findings suggest that genetic variation in *LARG* results in decreased LARG protein activity. Because LARG is one regulator of RhoA activity, decrease in LARG would predict decreased RhoA activation. Recently, it has been shown that activation of RhoA (and thus the Rho-kinase

TABLE 4—*Continued*

LARG-SNP12			LARG-SNP15				LARG-SNP18			
CC (n = 253)	TT + CT (n = 69)	P	AA (n = 126)	AG (n = 144)	GG (n = 52)	P	Tyr/Tyr (n = 100)	Tyr/Cys (n = 171)	Cys/Cys (n = 51)	P
144/109	43/26	0.42	79/47	78/66	31/21	0.36	63/37	97/74	29/22	0.57
32 ± 1	32 ± 1	0.46	32 ± 1	32 ± 1	32 ± 1	0.38	32 ± 1	32 ± 1	33 ± 1	0.49
125 ± 2	124 ± 4	0.98	120 ± 3	128 ± 3	127 ± 4	0.15	128 ± 3	124 ± 2	120 ± 5	0.22
2.2 ± 0.1	2.2 ± 0.1	0.43	2.2 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	0.43	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.1	0.40
0.40 ± 0.01	0.39 ± 0.01	0.42	0.41 ± 0.01	0.39 ± 0.01	0.38 ± 0.01	0.08	0.37 ± 0.01	0.41 ± 0.01	0.43 ± 0.02	0.0001
8.8 ± 0.1	8.5 ± 0.3	0.16	9.2 ± 0.2	8.6 ± 0.2	8.3 ± 0.3	0.008	8.3 ± 0.2	8.8 ± 0.2	9.5 ± 0.3	0.004

ROK) inhibits actions mediated through insulin receptor substrate and phosphatidylinositol 3-kinase/serine-threonine kinase signaling, which ultimately results in reduced skeletal muscle glucose transport (9). Decreased RhoA activation would therefore predict an increased rate of insulin-mediated glucose uptake (insulin sensitivity), which was observed in our study (Fig. 2). This is consistent with in vitro studies in 3T3-L1 adipocytes, showing that the level of glucose uptake activated by Clostridium botulinum C3 exoenzyme, specifically inactivating the small GTP-binding protein Rho, was similar to the level of glucose uptake activated by insulin, also pointing to a possible role of Rho in the regulation of glucose transport (35). It is, however, unexpected that the Tyr1306Cys was not associated with type 2 diabetes in our study (odds ratio 1.03 [95% CI 0.83–1.29] in an additive model). One possibility for the lack of association is that additional genes with larger effects on insulin action or genes that affect insulin secretion may be masking this association. Alternatively, because only 20 individuals were sequenced for variant detection, it is possible that a rare type 2 diabetes causative variant is present in *LARG* but was not detected.

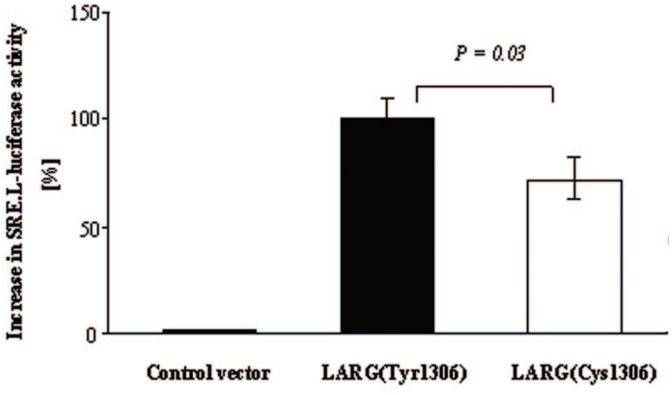

FIG. 1. Activation of the SRE, via serum response factors, in NIH3T3 cells by LARG plasmids. Plasmids pCGN-expressing LARG(Tyr1306) (Tyr at 1306) and LARG(Cys1306) (Cys at 1306) proteins were cotransfected into NIH3T3 cells with a luciferase reporter plasmid containing a SRE (SRE.L-luciferase) using LipofectAMINE Plus according to the manufacturer's protocol. Transfections were carried out in duplicate in six-well plates, and the data shown are the mean (±SE) of six experiments. Increase in luciferase activity was quantified relative to the control (pCGN without LARG) vector–induced level of the SRE-luciferase reporter system.

The Dbl homology and pleckstrin homology domains of GEFs are sufficient for nucleotide exchange activity. Studies of the atomic structures of the Dbl homology and pleckstrin homology domains of LARG alone and in complex with RhoA have shown that the Dbl homology/pleckstrin homology domains of LARG undergo a dramatic conformational change upon binding to RhoA, and both the Dbl homology and pleckstrin homology domains directly engage RhoA (36). Although the Tyr1306Cys is positioned COOH-terminal to these domains, any change that would modify interaction between these domains could potentially modulate Rho activation, and several cases of tyrosine phosphorylation–dependent activation of Rho-GEFs have been demonstrated (37–39). The best studied Rho-GEF is the Vav proto-oncogene, and the structural basis for relief of autoinhibition of its Dbl domain by tyrosine phosphorylation has been reported by Aghazadeh et al. (40). Furthermore, Chikumi et al. (41) showed that LARG and PDZ-RhoGEF could be tyrosine phosphorylated through focal adhesion kinase in response to thrombin, thereby enhancing the activation of Rho in vivo. In addition, a deletion form of PDZ-RhoGEF lacking the COOH terminus lost tyrosine phosphorylation ability, presumably as a result of a deleted phosphoacceptor site or structural feature necessary for substrate recognition (41). The structure of the LARG protein is highly related to that of PDZ-RhoGEF, and therefore, it is likely that similar mechanisms might be involved in tyrosine phosphorylation of the LARG. Because the Tyr1306Cys substitution is located in the COOH terminus of the LARG, it may be hypothesized that this substitution contributes to impaired phosphorylation of LARG and thereby results in decreased activity. However, this hypothesis needs further evaluations in studies focusing on phosphorylation of LARG. Consistent with Chikumi et al. (41), Suzuki et al. (27) demonstrated that tyrosine phosphorylation of LARG by Tec does not affect its basal RhoGEF activity but rather changes its regulation by G subunits and suggested that the modulation of the G12/13-RhoGEF pathway by tyrosine kinases may be a widely used mechanism for G-protein–coupled receptor–mediated Rho activation.

In conclusion, a Tyr1306Cys substitution in the *LARG* gene is highly associated with insulin action in nondiabetic Pima Indians. In addition, this amino acid substitution resulted in a decreased activity of the LARG protein. Based on these data, we suggest that this substitution may

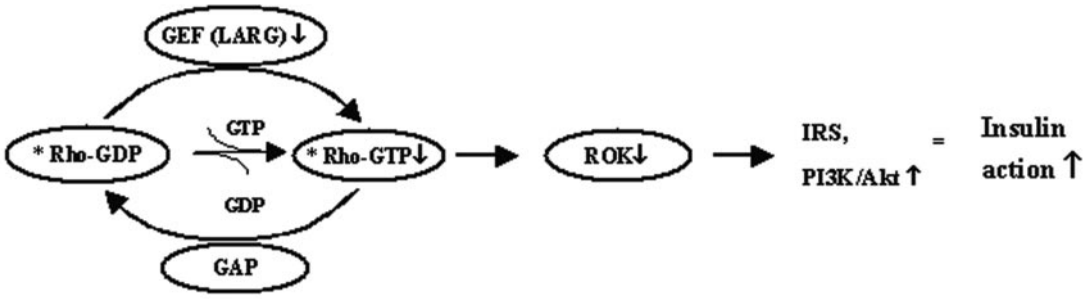

FIG. 2. Decreased activity of LARG caused by the Tyr1306Cys substitution is associated with higher skeletal muscle insulin sensitivity in Pima Indians. Rho proteins cycle between an inactive GDP-bound and an active GTP-bound state that is under the regulation of GEFs and GTPase-activating proteins (GAPs). Decreased LARG activity subsequently affects Rho-linked pathways. IRS, insulin receptor substrate; PI3/Akt, phosphatidylinositol 3-kinase/serine-threonine kinase Akt; ROK, Rho kinase.

lead to improved insulin sensitivity in Pima Indians through decreased LARG activity subsequently affecting Rho-linked pathways. Our findings provoke further studies necessary to clarify the impact of the Tyr1306Cys variation on insulin resistance and diabetes in other populations and to clarify the biochemical basis for the reduction in LARG activity. We believe that detailed investigation into LARG/Rho-related pathways might lead to revealing new targets for therapeutic approaches eventually helping to deal with burdens of insulin resistance and diabetes.

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