Rapid Publication

The Gene INPPL1, Encoding the Lipid Phosphatase SHIP2, Is a Candidate for Type 2 Diabetes In Rat and Man

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Genetic susceptibility to type 2 diabetes involves many genes, most of which are still unknown. The lipid phosphatase SHIP2 is a potent negative regulator of insulin signaling and sensitivity in vivo and is thus a good candidate gene. Here we report the presence of SHIP2 gene mutations associated with type 2 diabetes in rats and humans. The R1142C mutation specifically identified in Goto-Kakizaki (GK) and spontaneously hypertensive rat strains disrupts a potential class II ligand for Src homology (SH)-3 domain and slightly impairs insulin signaling in cell culture. In humans, a deletion identified in the SHIP2 3′ untranslated region (UTR) of type 2 diabetic subjects includes a motif implicated in the control of protein synthesis. In cell culture, the deletion results in reporter messenger RNA and protein overexpression. Finally, genotyping of a cohort of type 2 diabetic and control subjects showed a significant association between the deletion and type 2 diabetes. Altogether, our results show that mutations in the SHIP2 gene contribute to the genetic susceptibility to type 2 diabetes in rats and humans. *Diabetes* 51:2012–2017, 2002

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ARE, adenylate/uridylate-rich element; HA, hemagglutinin A; MAP, mitogen-activated protein; PKB, protein kinase B; QTL, quantitative trait locus; SH, Src homology; SV40, simian virus 40; UTR, untranslated region.

Recent data from knock-out mice (1) and in vitro studies (2–5) have identified type II SH2-domain–containing inositol 5-phosphatase, or "SHIP2," as a critical and essential negative regulator of insulin signaling and sensitivity. Indeed, decreased expression of SHIP2 and SHIP2 deficiency in mice leads to increased insulin sensitivity, whereas SHIP2 overexpression in various insulin-sensitive cell lines leads to decreased insulin signaling, i.e., insulin resistance. Given the importance of SHIP2 in the control of insulin sensitivity, we postulated that mutation(s) positively affecting SHIP2 activity, function, and/or expression might contribute to insulin resistance, a hallmark of type 2 diabetes.

RESEARCH DESIGN AND METHODS

Localization of the SHIP2 gene on rat chromosomes. Fluorescent in situ hybridization and radiation hybrids mapping were performed as described (6). The following forward and reverse primers were used to amplify a 140-bp DNA fragment of the rat SHIP2 gene from hybrid DNA: 5′-CCAGGGGT GAAAGTTTTGAG-3′ and 5′-CCTGACCCTGGGCCTAAAAG-3′.

SHIP2 gene amplification and sequencing in humans and rats. Consent was obtained from all subjects after the nature of the procedure was explained, and the investigation was conducted according to the principles expressed in the Declaration of Helsinki. All diabetic subjects were >35 years of age at diagnosis and met the World Health Organization's criteria defining diabetes status. The control subjects were randomly and anonymously chosen in a DNA library isolated from a large population of women consulting for a genetic diagnosis of mutation in the CFTR gene. The SHIP2 cDNA and gene sequences were obtained after PCR amplification. The sequencing products were run on an Applied Biosystem sequencer.

CHO-IR transfection, Akt/protein kinase B, and mitogen-activated protein kinase activities. CHO cells expressing the human insulin receptor were transfected as described (5), with the pcDNA3 vector (Invitrogen) or with the same vector containing either the wild-type or the R1142C-mutated hemagglutinin A (HA)–tagged SHIP2 cDNA (nucleotides 264–4360 from AF162781). After 2 days, cells were stimulated with 10 nmol/l insulin during 2 min and then lysed. The supernatant was assayed for Akt/ protein kinase B (PKB) and mitogen-activated protein (MAP) kinase activities as described (5). Each activity is calculated from triplicate ± SE.

Rat L6 myoblast and human 293 embryonic kidney cell transfection. Rat, mouse, simian virus 40 (SV40), and human 3′UTR were amplified by PCR and subcloned in pGEM-T easy. A DNA fragment containing the SV40 promoter and luciferase cDNA was excised from the pGL3 vector and introduced into the above plasmids. Each plasmid (0.8 μg/10 cm^2 well) was co-transfected in triplicate with 0.2 μg pSV–β-galactosidase transfection efficiency plasmid.

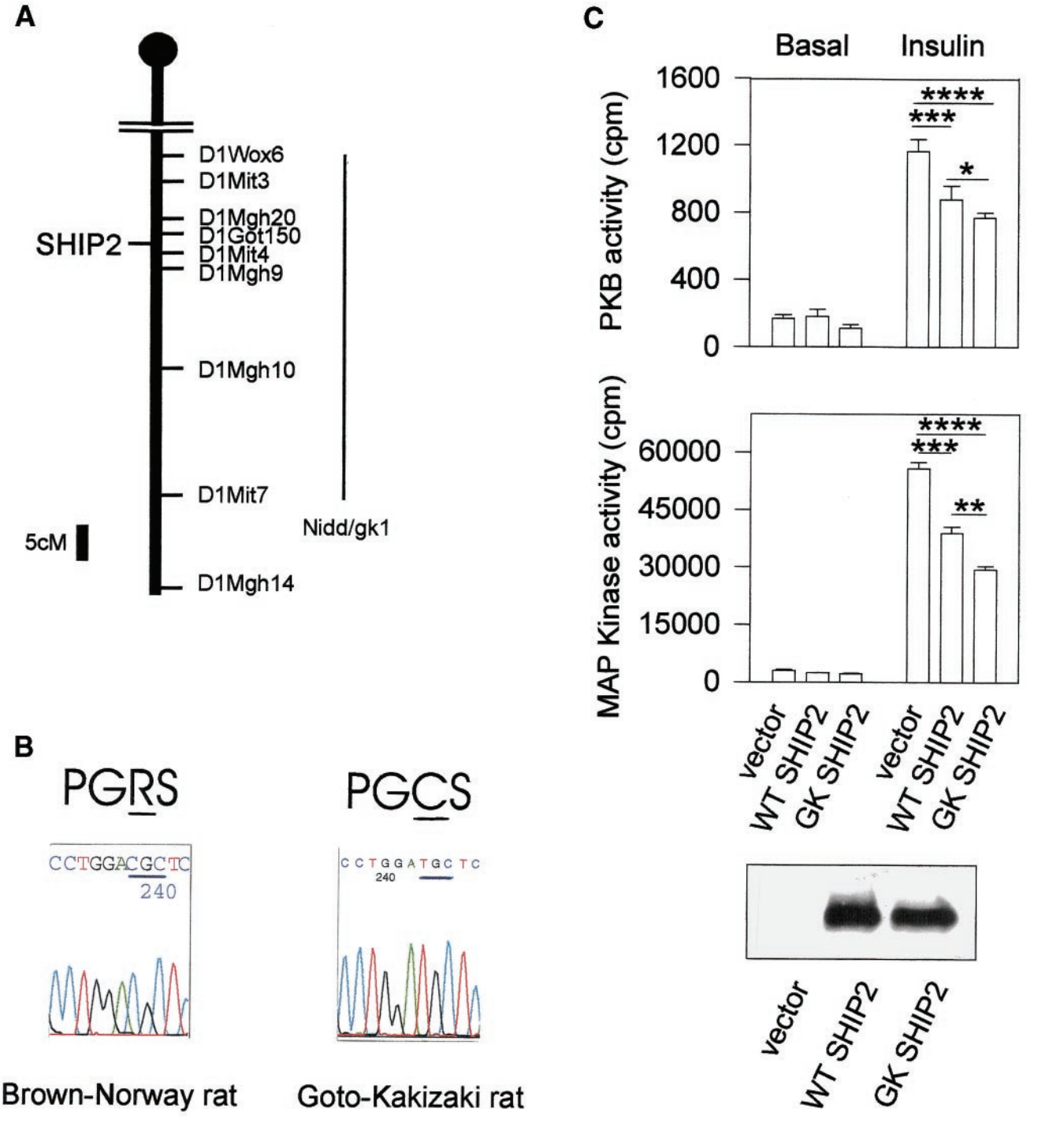

FIG. 1. The R1142C mutation in GK and SH rat strains: detection and analysis. *A*: Localization of the SHIP2 gene and the Nidd/gk1(GK rat) QTL on the long arm of rat chromosome 1. *B*: The R1142C mutation found in the GK and SH rat strains. *C*: Akt/PKB and MAP kinase activities in CHO-IR stimulated or not for 2 min with 10 nmol/l insulin. Results are the means of triplicate ± SE and are representative of a typical experiment out of three. Western blot analysis shows that the same amount of wild-type and mutated HA-tagged SHIP2 was expressed after transfection. *$P < 0.05$; **$P < 0.02$; ***$P < 0.002$; ****$P < 10^{-4}$ (by Student's t test).

After 24 h (human 293 cells) or 48 h (rat L6 myoblasts), the cells were lysed and processed either for luciferase activity according to manufacturer's instructions (Promega) or for total RNA extraction. The amount of β galactosidase present in the lysate was determined by enzyme-linked immunoassay (Boehringer Mannheim). Total RNA (1 μg/lane) was loaded on a 1% agarose gel and transferred to a nylon membrane. A 1.6-kb fragment of the luciferase cDNA excised from the pGL3 vector was used as a radiolabeled probe. Quantification of the signals was performed on a phosphorimager.

RESULTS AND DISCUSSION

We first localized the SHIP2 gene on rat chromosomes because chromosomal regions suspected to contain one or several genes predisposing to type 2 diabetes or insulin resistance have been identified using rat polygenic models (7–9). Fluorescent in situ hybridization (FISH) and radiation hybrid mapping revealed that the rat SHIP2 gene is

TABLE 1
cDNA sequence analysis around amino acid 1142 (SHIP2) or the corresponding SHIP1 cDNA region in various species

Name	Accession number		1142	
Class II ligand consensus		PXXPX	R	
Rat SHIP2	AB011439	P-GPG	R	SALLP
Rat SHIP2	AB025794	P-GPG	R	SALLP
Brown-Norway rat SHIP2*	—	P-GPG	R	SALLP
Zucker-Fatty rat SHIP2	—	P-GPG	R	SALLP
Goto-Kakizaki rat SHIP2	—	P-GPG	C	SALLP
SHR rat SHIP2§	—	P-GPG	C	SALLP
Mouse SHIP2	AF162781	P-GPG	R	SALLP
Human SHIP2	Y14385	PAGPA	R	SALLP
Rat SHIP1	U55192	PVKPS	R	SEMSQ
Mouse SHIP1	NM010566	PVKPS	R	SEMSQ
Human SHIP1	U57650	PIKPS	R	SEINQ

*As F344, COP, WF, WKY, WKY Leicester, SD, PD, Buffalo, Mib, LEW, BKI, BDII, LE, MNS, SS/jr, SR/jr and SPRD rat strains; §includes SHR/le, SHRSP/gla, and the original strain SHRSP/izm.

localized on the long arm of chromosome 1, at q33-36, between markers D1Got150 and D1Mit4 (Fig. 1*A*). It co-localizes with Nidd/gk1 quantitative trait locus (QTL), a QTL for glucose homeostasis identified in the diabetic GK rat strain (8). SHIP2 mRNA expression was investigated by Northern blotting in skeletal muscles from GK rats, but no significant difference was observed when compared with the diabetic and obese Zucker-fatty or control Brown-Norway strains (data not shown). Sequencing of the complete SHIP2 cDNA coding region in the above three rat strains revealed the presence of a unique mutation substituting an arginine to a cysteine at amino acid 1142 in the GK rat, within the proline-rich region of the protein (Fig. 1*B*, Table 1). The R1142C mutation identified in the GK rat was also present in the insulino-resistant and spontaneously hypertensive (SHR) rat, but not in 17 other rat strains tested (Table 1). This arginine is conserved in SHIP1 and SHIP2 proteins and might play an important functional role. Indeed, the surrounding amino acids match the consensus sequence for SH3 domain class II ligand (Table 1) (10,11). It has been shown that the conserved arginine present in class II ligands interacts with the third specificity pocket of the SH3 domain and that substitution by alanine results in decreased affinity and specificity (12). The COOH-terminal region of SHIP1 and SHIP2 is known to mediate interactions with protein partners and/or plasma membrane translocation (13–15).

To test the effects of the R1142C mutation on SHIP2 function in insulin signaling, wild-type and mutated SHIP2 cDNAs were introduced in CHO cells expressing the human insulin receptor (CHO-IR) by transfection. Because PKB/Akt and MAP kinase (ERK-2) are two signaling molecules downstream of the insulin receptor, their activities were analyzed in CHO-IR cells after hormone stimulation. Expression of wild-type or mutated SHIP2 cDNAs in CHO-IR cells resulted in decreased PKB and MAP kinase activities after insulin stimulation, as compared with vector-transfected CHO-IR cells (Fig. 1*C*). However, a slightly but significantly more important decrease was observed when the mutated protein was expressed.

Altogether, these data indicate that the R1142C mutation specifically identified in GK and spontaneously hypertensive (SHR) rats slightly impairs insulin signaling and may contribute to the genetic predisposition to type 2 diabetes and/or insulin resistance in these rat strains.

The presence of a relevant mutation associated with diabetes and/or insulino-resistance in two rat strains led us to investigate the human SHIP2 gene. The SHIP2 cDNA from eight unrelated Caucasian subjects with type 2 diabetes and from four control subjects of the same origin was sequenced. No difference was detected in the SHIP2 coding region. To our surprise, one of the type 2 diabetic subjects (SMH10), but none of the control subjects, exhibited a heterozygositic deletion in the proximal part of the SHIP2 3′UTR (Fig. 2*A*). The 16-bp deleted sequence contains an ATTTA pentamer potentially belonging to an adenylate/uridylate-rich element (ARE) (Fig. 2*B* and *C*). AREs are conserved sequence elements implicated in the regulation of messenger RNA (mRNA) stability and translation efficiency (16,17). Sequence comparison between mouse, rat, and human SHIP2 3′UTR identified ATTTA pentamers or related sequences, like ATTTTA hexamer, embedded in very conserved regions (Fig. 2*B* and *C*). In the rat and mouse 3′UTR, two pentamers and one hexamer sequences were detected (Fig. 2*B*). In man, a 16-bp fragment containing the first ATTTA pentamer was exactly duplicated, as compared with rat and mouse, and a total of three pentamers and one hexamer were detected (Fig. 2*B* and *C*). In addition, a very conserved thymidylate was mutated in adenylate in the hexamer, potentially affecting its function (Fig. 2*B*). To test the capacity of the SHIP2 rat, mouse, and human 3′UTR to regulate protein expression, plasmids containing the SV40 promoter followed by the luciferase cDNA and one of the 3′UTR described above were constructed and introduced in rat L6 myoblasts by transfection (Fig. 3*A*). The SV40 3′UTR was used as control, because it does not contain any AREs. The presence of the SV40 3′UTR downstream of the luciferase cDNA resulted in a 10- to 70-fold higher luciferase activity, as compared with SHIP2 rat, mouse, and human 3′UTR. In rat L6 cells, the lowest luciferase activity was detected with the rat 3′UTR, followed by the mouse and human 3′UTRs. A 4.5- and 7.7-fold increase in luciferase activity were respectively observed with these 3′UTR, as compared with the rat 3′UTR (Fig. 3*A*). Altogether, these results suggest that the AREs present in the SHIP2 3′UTR are functional and lead to decreased expression of the reporter gene. Thus, mutations in the SHIP2 3′UTR sequence could affect ARE function, SHIP2 expression, and insulin sensitivity, contributing to type 2 diabetes patho-

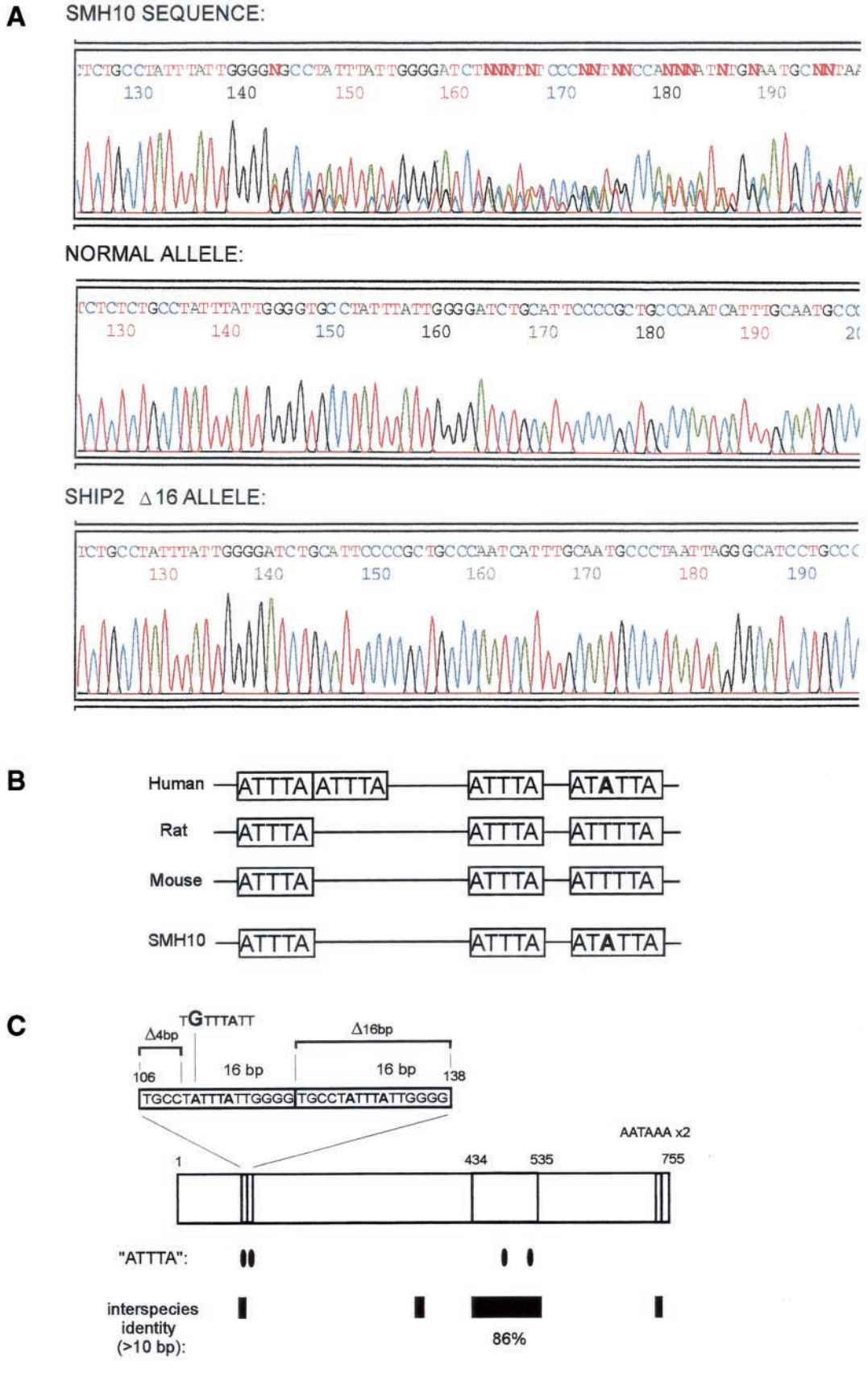

FIG. 2. The 3′UTR of the SHIP2 gene: structure and mutations. *A*: The Δ16-bp mutation found in the SHIP2 3′UTR from the SMH10 subject: sequence of the PCR product amplified from SMH10 cDNA (above), and sequences of the wild-type (middle) and the Δ16-bp mutant (below) after subcloning. *B* and *C*: Structure of the SHIP2 3′UTR: number and location of ATTTA pentamers and ATTTTA hexamers in man, rat, and mouse. Definition of the three mutations (Δ4bp, G/A substitution, and Δ16 bp) found in the SHIP2 3′UTR, and location of the regions with a high percentage of identity between mouse, rat, and man.

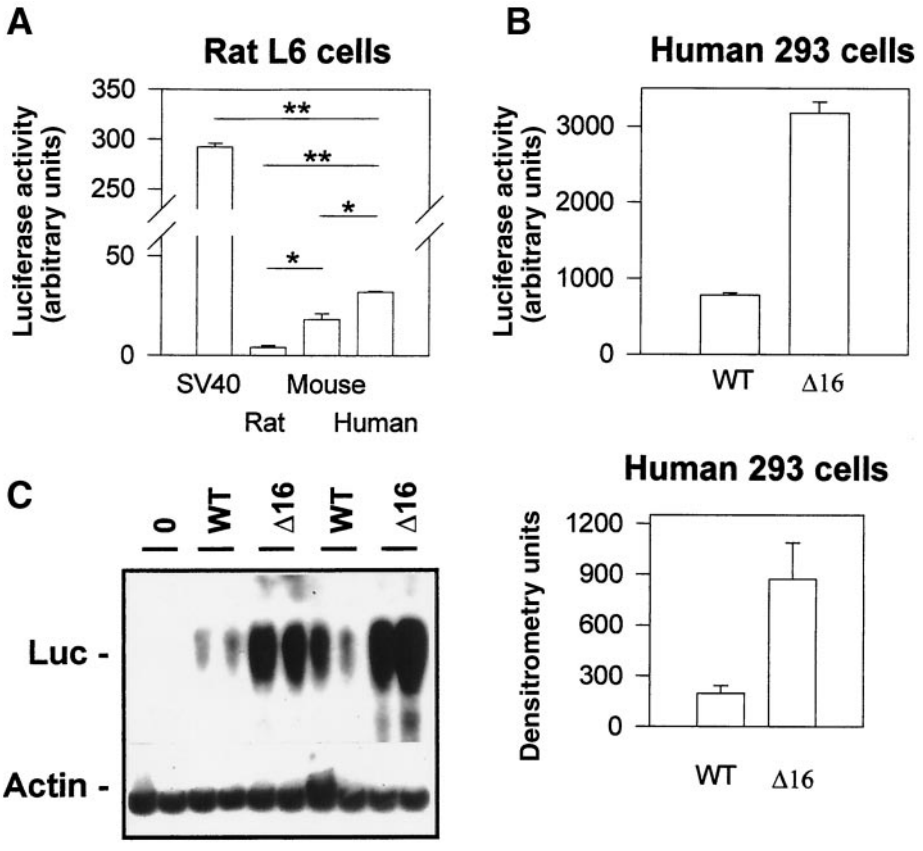

FIG. 3. Effects of the wild-type and the Δ16 SHIP2 3′ UTRs on luciferase reporter gene expression. Transfection efficiencies were assessed by β-galactidose protein quantification, and luciferase reporter gene expression was corrected accordingly. Differences in transfection efficiencies between the tested plasmids of the same experiment were always <10%. *A*: Luciferase expression in rat L6 cells transfected with a plasmid vector containing the 3′UTR from SV40, rat, mouse, or human SHIP2 gene. Results are the means ± SE and are representative of three separate experiments performed in triplicate. *$P < 0.03$; **$P < 0.005$ (Student's *t* test). *B*: Luciferase expression in human 293 cells transfected with a plasmid vector containing either the wild-type or the Δ16-bp mutation. Results are the means ± SE and are representative of four separate experiments performed in triplicate. *C*: Total RNA isolated from human 293 cells transfected with the empty plasmid vector (0) or a plasmid vector containing either the wild-type (WT) or the Δ16-bp mutated (Δ16) SHIP2 was analyzed with a luciferase or an actin probe. Densitometric analysis of the signals was performed with a phosphorimager. Results are the means ± SE.

genesis. To test the capacity of the mutated 3′UTR found in SMH10 type 2 diabetic subjects to regulate the gene reporter expression, the wild-type and Δ16 SHIP2 3′UTR were placed downstream of the luciferase cDNA, and the plasmids were introduced in human 293 cells by transfection (Fig. 3*B*). The presence of the Δ16 mutation resulted in a fourfold increase in luciferase activity (range 2.8–5.8; mean 4.0; $n = 4$; $P < 10^{-4}$ by Student's *t* test), as compared with the wild-type 3′UTR. The increased luciferase activity seen with the Δ16 mutant was not dependent on the presence of insulin: a similar ratio of the luciferase activities found with the wild-type and the Δ16 SHIP2 3′UTR was observed when human 293 cells were cultured with or without 100 nmol/l insulin (data not shown). The increased luciferase activity observed with the Δ16 mutant was associated with an equal increase in the luciferase mRNA level (Fig. 3*C*). Indeed, quantification of the luciferase mRNA signals found in transfected human 293 cells revealed a 4.5-fold increased level with the Δ16 mutant, as compared with the wild-type 3′UTR (range 3.4–5.1; mean 4.3; $n = 3$; $P < 0.02$ by Student's *t* test). Our results suggest that the Δ16 deletion identified in the SHIP2 3′UTR of one type 2 diabetic subject results in decreased ARE function and increased mRNA and protein expressions, which may result in decreased insulin signaling, i.e., insulin resistance in vivo. We next determined the accurate frequency of the Δ16 mutated allele in Caucasian populations of type 2 diabetic and control subjects. DNA samples from type 2 diabetic subjects originating from the U.K. ($n = 246$) and Belgium ($n = 169$), as well as from anonymous healthy individuals collected by the Genetics Department of the Erasme Hospital in Brussels ($n = 567$), were included in the experiment. A total of 9 Δ16 mutations (5 in the Belgian cohort and 4 in the English cohort) were identified in 415 unrelated type 2 diabetic subjects (frequency 2.16%), whereas only 3 mutations were identified in 567 control subjects (frequency 0.52%). Statistical analysis using the Pearson χ^2 test revealed a significant association between the Δ16 mutation and type 2 diabetes ($P = 0.021$). The nine diabetic subjects with a Δ16 mutation had a typical type 2 diabetes history: the age at diagnosis was always >40 years (range 44–67) and their treatment did not required insulin injections. BMI ranged from 23.9 to 38.1 kg/m^2. Five of the nine diabetic subjects had a familial history of type 2 diabetes, and seven of nine were hypertensive.

Sequence analysis identified two other mutations close to the same region in the cohort of diabetic subjects (Fig. 2*C*). A single nucleotide substitution (A to G) in the first ATTTA pentamer and a 4–base-pair deletion (Δ4) located

upstream of the same pentamer were present in three and one type 2 diabetic subjects ($n = 415$), respectively, but not in any of the control subjects tested ($n = 567$). Both mutations were associated with a slightly increased luciferase gene reporter expression in 293 cells (data not shown).

Together, these present data indicate that a 16-bp deletion including a potential ARE in the human SHIP2 3′UTR is significantly associated with type 2 diabetes, results in increased expression of reporter mRNA and protein in vitro, and presumably increased SHIP2 expression in vivo, leading to decreased insulin sensitivity. Thus, as in rats, SHIP2 is a candidate gene contributing to the genetic susceptibility of type 2 diabetes, a frequent metabolic disease characterized by the presence of insulin resistance and/or defects in insulin production/secretion by pancreatic β-cells. However, more detailed analysis, like family segregation of the present mutations, mutation frequency in larger diabetic cohorts from Caucasian and other ethnic groups, as well as SHIP2 protein quantification after muscle biopsy, are required to definitively validate SHIP2 as a predisposing gene for type 2 diabetes.

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REFERENCES

1. Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L, Schurmans S: The lipid phosphatase SHIP2 controls insulin sensitivity. *Nature* 409:92–97, 2001
2. Ishihara H, Sasaoka T, Hori H, Wada T, Hirai H, Haruta T, Langlois WJ, Kobayashi M: Molecular cloning of rat SH2-containing inositol phosphatase 2 (SHIP2) and its role in the regulation of insulin signaling. *Biochem Biophys Res Commun* 260:265–272, 1999
3. Sasaoka T, Hori H, Wada T, Ishiki M, Haruta T, Ishihara H, Kobayashi M: SH2-containing inositol phosphatase 2 negatively regulates insulin-induced glycogen synthesis in L6 myotubes. *Diabetologia* 44:1258–1267, 2001
4. Wada T, Sasaoka T, Funaki M, Hori H, Murakami S, Ishiki M, Haruta T, Asano T, Ogawa W, Ishihara H, Kobayashi M: Overexpression of SH2-containing inositol phosphatase 2 results in negative regulation of insulin-induced metabolic actions in 3T3–L1 adipocytes via its 5′-phosphatase catalytic activity. *Mol Cell Biol* 21:1633–1646, 2001
5. Blero D, De Smedt F, Pesesse X, Paternotte N, Moreau C, Payrastre B, Erneux C: The SH2 domain containing inositol 5-phosphatase SHIP2 controls phosphatidylinositol 3,4,5-trisphosphate levels in CHO-IR cells stimulated by insulin. *Biochem Biophys Res Commun* 282:839–843, 2001
6. Szpirer C, Van Vooren P, Rivière M, Scohy S, Collau G, Szpirer J: Localization of 54 rat genes, and definition of new synteny groups conserved in the human and the rat. *Mamm Genome* 11:729–735, 2000
7. Galli J, Li LH, Glaser A, Östenson CG, Jiao H, Fakhrai-Rad H, Jacob HJ, Lander ES, Luthman H: Genetic analysis of non-insulin dependent diabetes mellitus in the GK rat. *Nat Genet* 12:31–37, 1996
8. Gauguier D, Froguel P, Parent V, Bernard C, Bihoreau MT, Portha B, James MR, Penicaud L, Lathrop M, Ktorza A: Chromosomal mapping of genetic loci associated with non-insulin dependent diabetes in the GK rat. *Nature Genetics* 12:38–43, 1996
9. Aitman TJ, Gotoda T, Evans AL, Imrie H, Heath KE, Trembling PM, Truman H, Wallace CA, Rahman A, Doré C, Flint J, Kren V, Zidek V, Kurtz TW, Pravanec M, Scott J: Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat Genet* 16:197–201, 1997
10. Mayer BJ: SH3 domains: complexity in moderation. *J Cell Sci* 114:1253–1263, 2001
11. Kay BK, Williamson MP, Sudol M: The importance of being a proline: the interaction of proline-rich motifs in signalling proteins with their cognate domains. *FASEB J* 14:231–241, 2000
12. Feng S, Chen JK, Yu H, Simon JA, Schreiber SL: Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. *Science* 266:1241–1247, 1994
13. Aman MJ, Walk SF, March ME, Su HP, Carver DJ, Ravichandran KS: Essential role for the C-terminal noncatalytic region of SHIP in FcgammaRIIB1-mediated inhibitory signaling. *Mol Cell Biol* 20:3576–3589, 2000
14. Damen JE, Ware MD, Kaleshnikoff J, Hugues MR, Krystal G: SHIP's C-terminus is essential for its hydrolysis of PIP(3) and inhibition of mast cell degranulation. *Blood* 97:1343–1351, 2001
15. Dyson JM, O'Malley CJ, Becanovic J, Munday AD, Berndt MC, Coghill ID, Nandurkar HH, Ooms LM, Mitchell CA: The SH2-containing inositol polyphosphate 5-phosphatase, SHIP-2, binds filamin and regulates submembraneous actin. *J Cell Biol* 155:1065–1080, 2001
16. Chen CA, Shyu AB: AU-rich elements: characterization and importance in mRNA degradation. *Trends Biochem Sci* 20:465–470, 1995
17. Bakheet T, Frevel M, Williams BRG, Greer W, Khabar KSA: ARED: human AU-rich element-containing mRNA database reveals an unexpectedly diverse functional repertoire of encoded proteins. *Nucl Acids Res* 29:246–254, 2001