

## Mutations in the glucokinase regulatory protein gene in 2p23 in obese French caucasians

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### Abstract

**Aims/Hypothesis.** Glucokinase regulatory protein (GKRP) controls the activity of glucokinase in liver but possibly also in some areas of the central nervous system, suggesting that it could play a role in body mass control. Its gene is located in a region (2p21–23) linked to serum leptin levels. Our goal was to investigate whether mutations in the GKRP gene were associated with obesity.

**Methods.** Mutations were sought in the GKRP gene of 57 patients from the families of the French genome-wide scan for obesity that contributed most to the positive LOD score with 2p21–23. The identified mutations were further sought in 720 unrelated obese individuals and 384 individuals of normal weight and their effect on the properties of recombinant GKRP were investigated.

**Results.** The most frequent mutation (Pro446Leu) had a similar allele frequency in the obese (0.63) and normal weight (0.64) subjects and did not affect the prop-

erties of GKRP. Similarly, no effect on the properties of GKRP was observed with Arg590Tyr, found in 10 out of 720 obese subjects and in 2 out of 384 control subjects ( $p=0.18$ ). Mutation Arg227Stop was found in one obese family and in 1 out of 384 control subjects and led to an insoluble protein. Mutation Arg518Gln, replacing a conserved residue, led to a marked decrease in the affinity of GKRP for both fructose 6-phosphate and fructose 1-phosphate and to a destabilization of GKRP. However, this mutation did not co-segregate with obesity in the single family in which it was found.

**Conclusions/interpretation.** Mutations that affect the properties of GKRP are found in the French population, but they do not seem to account for the linkage between the 2p23 locus and quantitative markers of obesity. [Diabetologia (2003) 46:704–711]

**Keywords** Obesity, mutations, glucokinase, regulatory protein, gene, 2p21–23.

Obesity is a major risk factor for several life-threatening diseases such as Type 2 diabetes, hypertension, dyslipidaemia and coronary heart disease [1]. It is a multifactorial disorder with a strong genetic component, as indicated by its familial clustering and the

high concordance of body composition in monozygotic twins [2]. Furthermore, several syndromic or non-syndromic forms of obesity are now known to be due to mutations in a single gene, such as those encoding leptin [3], the leptin receptor [4], proopiomelanocortin (POMC) [5], prohormone convertase 1 (PC1) [6] and the melanocortin-4 receptor (MC4-R) [7, 8]. Taken together, these monogenic forms only account for a small percentage of obesity, which in most cases is probably due to the interaction of environmental factors with several predisposing genes.

Several genome scans for human obesity allowed the identification of various regions linked to obesity or to quantitative traits related to obesity. This is the case for the 2p21–23 locus, which showed a positive

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**Abbreviations:** GKRP, glucokinase regulatory protein.

linkage to leptin levels and fat mass in different populations [9, 10, 11]. Several genes at this locus that could potentially play a role in body weight homeostasis have been screened in French Caucasians. They include the *urocortin 1* gene [12], which encodes a protein that binds to the same receptor as CRF, a factor known to have an anorexigenic effect [13], and the *POMC* gene [14], which encodes a protein precursor that in some cell types is converted to alpha-MSH, also known to have anorexigenic effects [5]. The genetic variants found for these two genes do not explain the linkage between leptin levels and the 2p21–23 locus observed in the genome-wide scan carried out in a cohort of the French population [10].

The brain regulates energy homeostasis by balancing energy intake, expenditure and storage. To achieve this it has specialised neurons that receive and respond to both metabolic and afferent neural signals that communicate information about the energy status of the body [15, 16]. An attractive hypothesis in this context is that glucokinase could play a role in a central glucose-sensing mechanism [17, 18]. Glucokinase, an enzyme present in liver and in pancreatic islets, has a low affinity for glucose, which allows it to play the role of a glucose sensor in beta cells of pancreatic islets [19]. The finding that glucokinase is present in brain regions, most particularly the hypothalamus [20, 21], that contain glucose-sensitive and glucose-responsive neurons [22] suggests that this enzyme could play a similar role in brain as in insulin-secreting cells.

In the liver, glucokinase activity is controlled by a protein (glucokinase regulatory protein, GKR) that acts as an inhibitor in the presence of fructose 6-phosphate. The effect of GKR is suppressed by the specific fructose metabolite, fructose 1-phosphate [23, 24]. Recent data indicate that GKR is also present in the central nervous system [25], suggesting that it could also modulate glucokinase response to glucose in the central nervous system. This, together with the fact that the GKR gene is located in 2p21–23 [26, 27] led us to search for mutations in the *GKR* gene in obese patients from the families that had shown a linkage between the level of leptin and the region of chromosome 2 where the *GKR* gene is localised [10]. We identified some potentially important mutations and evaluated their role by linkage disequilibrium analysis and by testing their effect on the properties of recombinant GKR. The aim of this study is to report these findings.

## Subjects and methods

*Choice of obese patients included in the primary screening.* The 57 unrelated French Caucasian obese patients (mean BMI:  $40.83 \pm 8.6$  kg/m<sup>2</sup>) were selected from families contributing to the suggestive linkage of their obesity to the p21–23 locus on

chromosome 2, as assessed with marker D2S165. This marker is about 0.8 Mbp centromeric to the GKR gene, according to the current version of the human genome sequencing project (available at <http://www.ncbi.nlm.nih.gov>). The genetic cause for their obesity was unexplained and the other two obvious candidate genes (coding for POMC and urocortin-1) in this region of chromosome 2 had already been screened for mutations. There was no evidence for a linkage between obesity and the genetic variants found in these two genes [12, 14]. Informed consent was obtained from the subjects participating in the study, which was carried out in accordance with the principles of the Declaration of Helsinki.

*Mutation detection.* Single-strand conformation polymorphism (SSCP) [28] and heteroduplex [29] analysis were used to screen all 19 exons, as well as the flanking intron sequences of the human GKR gene. PCR reactions were carried out on 15 ng genomic DNA in 10  $\mu$ l volumes using the appropriate primers (Table 1). The SSCP and heteroduplex protocols were as previously described [30]. The PCR products showing a shift in SSCP or heteroduplex were sequenced directly on both strands on a CEQ2000 sequencer (Beckman, Fullerton, Calif., USA) using the manufacturers' protocol. The PCR products were purified before sequencing using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

The genotyping of the variants identified in the primary screening was carried out in 720 unrelated obese individuals (mean age:  $49.3 \pm 14.7$  years; BMI:  $34.8 \pm 3.9$  kg/m<sup>2</sup>) and in 384 non-obese, non-diabetic control subjects (mean age:  $56.8 \pm 13.5$  years; BMI:  $22.9 \pm 2.4$  kg/m<sup>2</sup>) by Light Cycler technology (Roche Molecular Biochemicals, Penzberg, Germany) [31]. Among 541 of these obese subjects, for whom information on the blood glucose level was available, 101 (18.7%) had Type 2 diabetes (fasting glycaemia  $\geq 7$  mmol/l or glycaemia 120 min after a glucose load  $\geq 11.1$  mmol/l) and 123 (22.7%) were glucose intolerant (fasting glycaemia  $< 7$  mmol/l and glycaemia at 120 min between 7.8 and 11.1 mmol/l).

*Site-directed mutagenesis of GKR.* Except for the Arg227Stop mutant, the mutants of rat liver GKR were prepared with a PCR-based technique using back-to-back primers [32], Pwo polymerase and the expression vector of rat liver GKR as a template [33]. The expression plasmid for the Arg227Stop mutant was constructed by PCR amplification of a rat liver cDNA using one primer that contained the initiator ATG flanked at its 5'-end by a NdeI restriction site and a second one that replaced the Arg227 codon by a TGA terminator flanked at its 3'-end by a BamHI restriction site. The amplification product was phosphorylated, subcloned into the EcoRV site of pBluescript II KS (Stratagene, La Jolla, Calif., USA) and finally cloned between the NdeI and BamHI restriction sites of the pET3a expression vector [34]. All five mutant plasmids were sequenced to rule out any PCR errors before transformation of *E. coli* BL21(DE3) pLysS and expression of the recombinant proteins.

*Expression, purification and GKR assays.* The proteins were expressed at 18°C in M9 salts medium and partially purified by polyethylene glycol precipitation and DEAE-Sepharose chromatography [33]. The purity of GKR in the DEAE-Sepharose pools was about 70% and its concentration was calculated by multiplying the total concentration of protein by the purity factor. GKR was assayed based on its ability to inhibit recombinant human liver glucokinase in the presence of 5 mmol/l glucose and the indicated concentrations of fructose 6-phosphate or fructose 1-phosphate, using a pyruvate kinase/lactate dehydrogenase coupled assay [33, 35].

**Table 1.** Primers used for SSCP/heteroduplex analysis

Amplified region	Forward primer <sup>a</sup>	Reverse primer <sup>a</sup>	Fragment length (bp)
Exon 1	TCAGATAAGGCCCTGCCAACTGGAA <sup>b</sup>	TCAGCACAGAAAGCCAACATGAAG	195
Exon 2	TGAGGCTTTGGCTTCCCTGCTCCAT	TATTTCCAGGGTCCAGGTCTTGGG	236
Exon 3	CTAATATCGGAAACCCTGAGACCC	CAGAAAAGTTTAGGCAGGGAACAG	190
Exon 4	TGAGCCAGGCCCTGGTAATATATG	TCTTTATGGTCTGAGGGTCACCT	177
Exon 5	CCAAGATAATTGTTTCTTCTGTCAT	ATTTAGGTCAAACAAAATATTCATAGGC	173
Exon 6	ATAGGCTTCTGCTTCCCAGCCACT	GAGGTCTCTGGTCAATGTCAGTGA	154
Exon 7	CCTGGAATCCTCCCAGTTACTACTA	CTCTCTAAAATTGATCCACTCAACC	141
Exon 8	TGGCACCCTCAGAGTAATGACC	AGGGCTGCCAGTCATTCCAGAA	183
Exon 9	GGCCCTTCTTTGAGATCCTCATAG	GTGTTGGAGGGGTGGAGAACATAG	207
Exon 10	AATTCCTCTTGGGTGTCTTCACCT	CTGAGCTCTCTTATCCAGATCC	180
Exon 11	AATTGGGCCCTTCTTGAGAGCTG	GGTCACATCCTCACCTCTAAACAC	174
Exon 12	GCACGGTGATCTCTCATGTCCTGA	GGAAGCAGGAAGAACATCTGGACT	174
Exon 13	CCTTTCCTATATAGCCAATCACTCC	AGCTTCCCCAAGTCTGTTCTTCT	157
Exon 14	TAGAGTGTTCCTCATGGGAGGGACC	ACAACAGCTCCTCTGAACCATTCTA	211
Exon 15	ACAGCCAGCCTTTCGACTCTGATG	AAGTCCACCTGGGTCCCTTTGT	179
Exon 16	AGCCAGGCCCTTCTGGAGATGCCT	TGAGCCCTTACTGCAGATAGGAC	176
Exon 17	AGGGCCCTGTTCACTCTGCTGCTT	CTCCTCTGGCAGCCTAAGCATTGA	245
Exon 18	GGCCTTTAGCCTGACACTGATCTT	TGTTGAATGACCAGCAGTGGGCAG <sup>b</sup>	204
Exon 19	TCCGGGGTCTTTCTCTGATGACC <sup>b</sup>	GCTGAAGTGGCAGGGTTGGC	250

<sup>a</sup> Primers are intronic, unless noted otherwise

<sup>b</sup> Exonic primer

**Expression and detection of GGRP in HEK 293 cells.** The coding sequences of the wild-type and of the Arg518Gln mutant of rat liver GGRP, flanked by 12 nucleotides of the 5' untranslated region (CAGTGTGGGACC) or by an "optimal" Kozak consensus sequence (CCGCCGCCACC) [36], were introduced into the eucaryotic expression vector pcDNA3.1/Zeo (Invitrogen, Life Technologies, Merelbeke, Belgium) between the NheI and the BamHI restriction sites. The resulting plasmids were used to transform *E. coli* DH5 alpha cells. The plasmid DNA was amplified and purified using a Plasmid Midi Kit (Qiagen). HEK 293 cells were cultured in 10-cm diameter dishes in Dulbecco's minimal essential medium containing 10% (v/v) foetal calf serum and transfected using a modified calcium phosphate procedure [37]. The dishes contained 80% confluent HEK 293 cell cultures and each of them was transfected with 7.5 µg of one of the four pcDNA 3.1 constructs or a control plasmid containing the cDNA encoding green fluorescence protein (GFP), used as a control for transfection. Each transfection experiment was done in triplicate. The cells were incubated overnight at 37°C and the next morning the medium was aspirated, dead cells were removed by washing with PBS and fresh Dulbecco's minimal essential medium, containing 10% (v/v) foetal calf serum, was added. The cultures were then left at 37°C for 16 h or 48 h to overexpress wild-type or Arg518Gln GGRPs. The cells were harvested by removing the medium, washed with 5 ml of PBS and resuspended in 1 ml of lysis buffer (20 mmol/l Hepes pH 7.1, 1 mmol/l dithiothreitol and 5 mg/l leupeptin and antipain). To extract the soluble proteins, the cell suspension was submitted to three cycles of freezing and thawing followed by a 30-min centrifugation at 12 000×g at 4°C. The supernatant was removed and the pellet was re-suspended in 1 ml of lysis buffer. The overexpression of GGRP in both soluble and insoluble fractions was visualised by Western blots, which were quantitated using the NIH Image analysis software (<http://rsb.info.nih.gov/nih-image/>).

Western blots were prepared [38] after SDS-PAGE of 5 µg of total protein from the extracts of the HEK 293 cell cultures

that overexpressed wild-type GGRP, the Arg518Gln mutant or the control GFP plasmid. Prior to electrophoresis, we added to our samples 35 µg of protein from a homogenate of rat brain (which does not express a detectable amount of GGRP in our procedure). This increased the resolution of the gel by blocking non-specific interactions between the proteins in the samples and the polyacrylamide. The antiserum used was prepared by injecting subcutaneously New Zealand rabbits (Charles River laboratories, Brussels, Belgium) with 100 µg of purified recombinant rat GGRP [33] in Freund's complete adjuvant. The injections were repeated four times at 2-week intervals. The detection method used was the enhanced chemiluminescence detection system [39].

**Statistical analysis.** A *p* value less than 0.05 was considered statistically significant.

## Results

**Search for mutations in the GGRP gene.** The human GGRP gene has 19 exons and encodes a 625 amino acid residue protein. Mutations were sought in the GGRP gene by SSCP/heteroduplex analysis of all 19 exons of GGRP in 57 unrelated French obese patients. Three novel mutations were found: Arg227Stop (once), Arg518Gln (once), His590Tyr (twice) in addition to Pro446Leu (43 times) and an intronic polymorphism in intron 16 [c.1489-22 (C->G)] (8 times). The position of this last nucleotide change at some distance from the splice site suggests that it is most likely a non-functional polymorphism. This mutation was therefore not further studied. A larger scale screening of the four mutations was carried out among 720 unrelated obese subjects and 384 control subjects (Table 2).

**Table 2.** Mutations found in the human GKR P gene

Localisation	Nucleotide change <sup>a</sup>	Amino acid change	Residue conservation	Frequency in primary screening	Frequency in obese subjects	Frequency in control subjects
Exon 9	C745T	Arg227Stop	Not relevant	1/57	1/720	1/384
Exon 15	C1403T	Pro446Leu	Semi-conserved <sup>b</sup>	43/57 (27 P/L; 16 L/L)	454/720 (335 P/L; 119 L/L)	246/384 (188 P/L; 58 L/L)
Intron 16	c.1489-22 (C->G)	none		8/57	not determined	not determined
Exon 17	G1619A	Arg518Gln	Extremely conserved	1/57	1/720	0/384
Exon 19	C1834T	His590Tyr	Non-conserved	2/57	10/720	2/384

<sup>a</sup> Nucleotides are numbered according to the cDNA sequence reported by [27] (accession number: 001486)

<sup>b</sup> Conserved in other vertebrates but not in bacteria.

#### A. Pro446Leu

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X-GKRP  HATAGQYLPN  SLKKTIPSII  456
R-GKRP  HSTVGQSLPA  PLKKLFPSLI  457
H-GKRP  HSTVGQTLPI  PLKKLFPSII  457
          L
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#### B. Arg518Gln

```
HI-Yfeu ASNEKLRKARA  VRIVMQATDC  254
X-GKRP  INNSKLFHRA  TSVLQRLTGQ  528
R-GKRP  IANSKLFWRA  LAMLQRFSGQ  529
H-GKRP  ISNSKLFWRA  LAMLQRFSGQ  529
          Q
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#### C. His590Tyr

```
HI-Yfeu STLSKSEAKV  LLERHQGKLR  299
X-GKRP  RSCTIQDSRS  RINSSLSIRS  598
R-GKRP  LRCSISEAKA  RLSAASVCE  599
H-GKRP  FRCSITEAQA  HLAAAPSVCE  599
          Y
```

**Fig. 1.** Partial sequence alignment of GKRP s with the Haemophilus influenzae homolog. The following sequences are shown: Xenopus (X), rat (R) and human (H) GKRP s; H. influenzae Yfeu (HI Yfeu). Residues that are conserved between the sequences are shown in boldface. Mutated residues are shown below the alignment. Numbers indicate the position of the last amino acid in the shown sequences

The most frequent mutation is Pro446Leu. It corresponds to the replacement of a residue conserved in rat and Xenopus GKRP s, in a region that does not, however, align with the bacterial homologs of GKRP (Fig. 1). There was no difference in the prevalence of this mutation (whether in the homozygous state or the heterozygous state) between obese and normal weight subjects (control: 138 Leu/Leu; 188 Pro/Leu; 58 Pro/Pro i.e. 36.9, 46.5 and 16.5%; obese: 266 Leu/Leu; 335 Pro/Leu; 119 Pro/Pro, i.e. 35.9, 49.0 and 15.1%;  $p=0.705$  in the  $\chi^2$  test). Furthermore, there was no statistically significant difference ( $\chi^2$  test) in the prevalence of this mutation between the three subcategories (normoglycaemic, mildly hyperglycaemic and Type 2 diabetic subjects) of the obese control group. As a mean, the blood insulin levels were (means  $\pm$  SD)  $12.62 \pm 10.22$ ,  $12.58 \pm 10.46$  and  $14.56 \pm 8.07$   $\mu$ U/ml

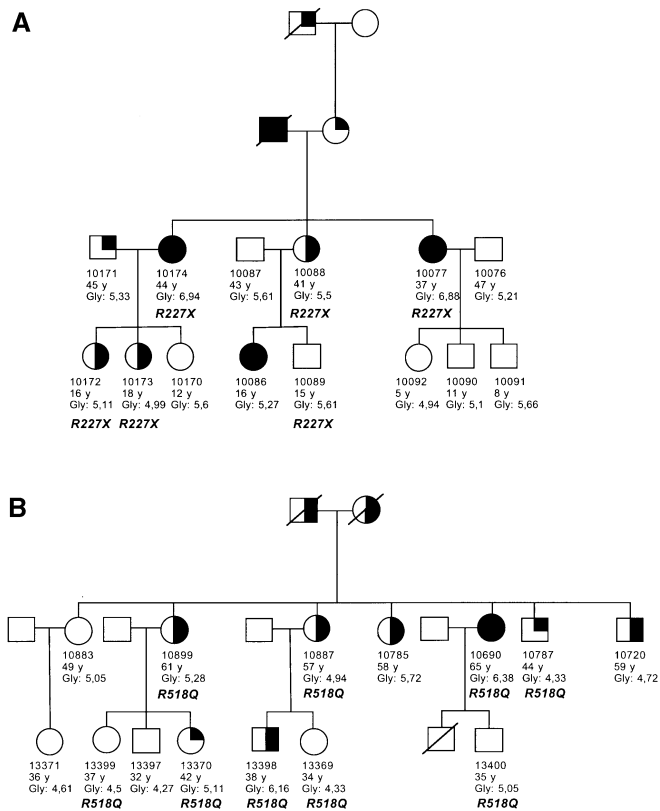
for the Pro/Pro ( $n=120$ ), Pro/Leu ( $n=202$ ) and Leu/Leu ( $n=80$ ) group, respectively; these values were not different (Students  $t$  test) ( $p>0.05$ ).

The next most frequent mutation is His590Tyr. It corresponds to the replacement of a non-conserved residue in a rather non-conserved region (Fig. 1) and was initially found twice in the screening of the 57 obese patients. In the two control populations, it was found in 10 out of 720 obese subjects and in 2 out of 384 control subjects ( $p=0.185$ ). Intra-familial analyses of pedigrees in which obese individuals harbour this mutation indicated no co-segregation with obesity (data not shown).

The Arg227Stop mutation was initially found in one obese subject. It was not identified in other obese subjects, but was found once in the control group. In the unique obese family carrying this mutation, five out of the six people bearing it are obese whereas one non-obese subject does not carry the mutation (Fig. 2A). The family of the non-obese control carrying the Arg227Stop mutation was also analysed; none of the mutated individuals were obese (data not shown) indicating that this mutation does not predispose to obesity.

The Arg518Gln mutation, which corresponds to the replacement of a conserved residue (Fig. 1), was found in only one obese subject and was absent in both obese and non-obese control groups. This mutation was found in six obese family members, and also in three subjects of normal weight, suggesting that it did not co-segregate with obesity (Fig. 2B).

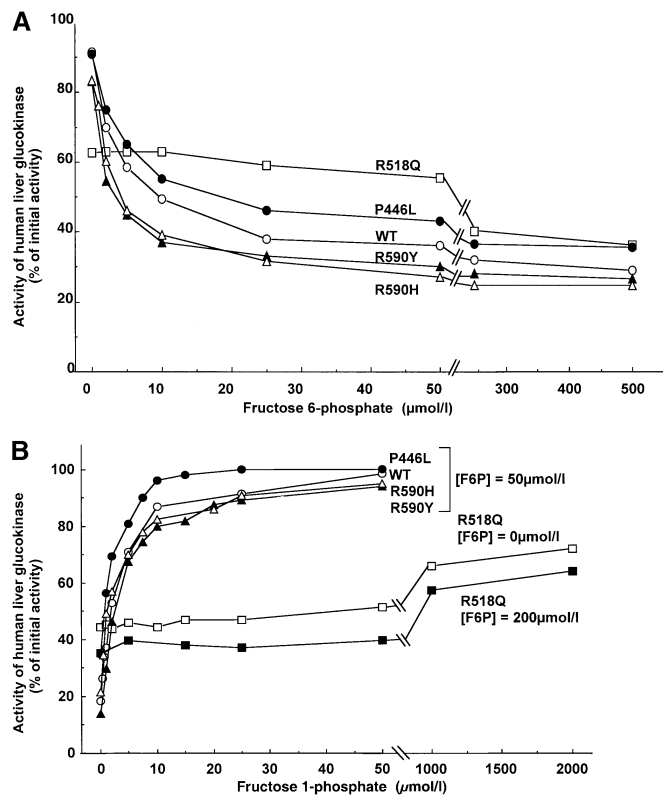
*Functional study of the mutations.* Rat liver GKRP can be prepared by overexpression in *E. coli*. Several mutants have been prepared in this way in sufficient amounts to achieve their biochemical characterisation. Unfortunately, for unknown reasons, the same expression system does not work with human GKRP, the yield of soluble protein being extremely low. The mutations that were found in the human GKRP gene were therefore introduced into the rat GKRP sequence to study their effect. It is indeed likely in view of the high degree of conservation between the two proteins (87% sequence identity), that the investigated muta-



**Fig. 2A, B.** Co-segregation analysis of obesity and GKRP mutations Arg227Stop (**A**) and Arg518Gln (**B**) in two French Caucasian families. Filled circles represent “super-obese” individuals (BMI>40 kg/m<sup>2</sup>), half-filled circles represent obese individuals (30 kg/m<sup>2</sup><BMI<40 kg/m<sup>2</sup>), quarter-filled circles represent overweight individuals (27 kg/m<sup>2</sup><BMI<30 kg/m<sup>2</sup>) and empty circles represent non-obese individuals. The first line under the symbols is the identification number, the second line the age of the exam and the third line the fasting blood glucose (in mmol/l). All numbered individuals have been screened for the mutations

tions have the same effect on both rat and human proteins. Since His590 is not conserved in the rat (where it is replaced by an arginine, Fig. 1), two different mutants (Arg590Tyr and Arg590His) were produced to investigate the effect of the human mutation His590Tyr.

All proteins were produced in *E. coli*, yielding partially soluble proteins in the case of the substitutions. In contrast, the protein with a premature stop codon at position 227 was found to be completely insoluble, suggesting that it behaves as a null mutant. The soluble proteins were partially purified by poly(ethylene-glycol) precipitation followed by chromatography on DEAE-sepharose. All four point mutant proteins behaved as inhibitors of glucokinase with similar potencies (not shown), 50% inhibition being observed at about 4 µg/ml. In the case of mutants Pro446Leu, Arg590Tyr and Arg590His, the regulation by fructose 6-phosphate and fructose 1-phosphate was not different from that observed with the wild-type protein, the inhibition being greatly reinforced by fructose 6-phos-

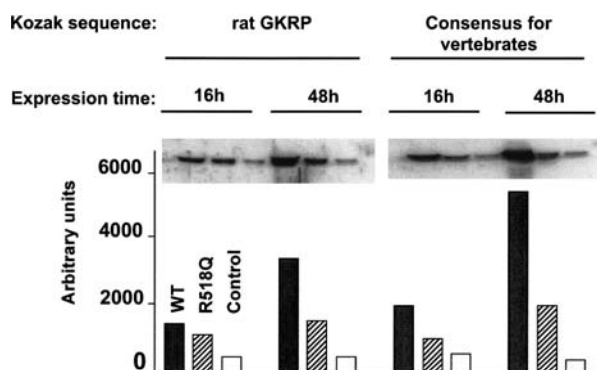


**Fig. 3A, B.** Effect of mutations on the sensitivity of GKRP to fructose 6-phosphate (**A**) and fructose 1-phosphate (**B**). Glucokinase activity was measured in the presence of 10 mg/l wild-type or mutant GKRP, 5 mmol/l glucose and (**A**) the indicated concentrations of fructose 6-phosphate or (**B**) 0, 50 or 200 µmol/l fructose 6-phosphate and the indicated concentrations of fructose 1-phosphate. Wild-type GKRP (white circles), Pro446Leu (black circles), Arg518Gln (white squares), Arg590His (white triangles), Arg590Tyr (black triangles)

phate and antagonised by fructose 1-phosphate (Fig. 3). In contrast, mutant Arg518Gln showed several distinct properties: (i) it was much less dependent on fructose 6-phosphate for its effect on glucokinase; (ii) its affinity for fructose 6-phosphate was considerably decreased (Fig. 3A) and (iii) this mutant had also a decreased affinity for fructose 1-phosphate (Fig. 3B), for which a dissociation constant of 1.6 mmol/l was calculated, as compared to about 1 µmol/l for the wild-type protein.

The heat-stability of the various point mutants was also investigated. Mutant Arg518Gln was found to be much more unstable than the wild-type protein and the other mutants: it was about 70% inactivated by a 20 min incubation at 40°C whereas the wild-type and the other mutant proteins kept more than 90% of their initial activity under these conditions.

To assess the effect of this heat instability in a mammalian cell context, HEK 293 cells were transfected with constructs containing rat wild-type GKRP and the Arg518Gln mutant. We tested two types of



**Fig. 4.** Expression of wild-type and Arg518Gln GKR in HEK 293 cells. HEK 293 cells were transfected with constructs encoding wild-type rat liver GKR or the Arg518Gln mutant. Cells were harvested 16 h and 48 h after transfection and analysed by western blotting. One representative experiment out of five is shown

constructs, one containing upstream of the ATG, the 12 nucleotides in the rat GKR cDNA, and a second one where this sequence was modified in order to obtain an “optimal” consensus Kozak sequence. Cells were extracted and analysed by western blotting with an antibody directed against recombinant rat liver GKR (Fig. 4). A background of GKR was detectable in the HEK 293 cells. Transfection of the constructs encoding the wild-type protein resulted in a time-dependent increase in the amount of immunodetectable GKR. A lower increase was observed with the Arg518Gln mutant and the difference was more pronounced at 48 h than at 16 h, consistent with a decrease in protein stability.

## Discussion

The objective of this work was to test the hypothesis that mutations in the GKR gene, a positional candidate, might be involved in the susceptibility to obesity. Four mutations that affect the coding sequence have been identified. One of them, Pro446Leu, that was previously found in the Scottish population [40], is frequent. The fact that its frequency is the same among obese subjects as in normal weight subjects indicates that it does not play a role in the genetic risk for obesity, at least in the French population. The same conclusion applies to mutation His590Tyr, which is probably an infrequent polymorphism without pathophysiological significance.

Mutation Arg227Stop removes about two thirds of the length of the protein. It does not seem to be compatible with proper folding and it must therefore be considered as a null mutation. It might be associated with obesity in one family but certainly not in another one, which advocates against it having a role in the development of obesity.

The most interesting mutation in terms of structure/activity relationship is Arg518Gln. GKR is distantly homologous to glucosamine 6-phosphate synthase, which like GKR, is able to bind fructose 6-phosphate. The homology between GKR and glucosamine 6-phosphate synthase has allowed the identification of regions in GKR that participate in fructose 6-phosphate and fructose 1-phosphate binding [33]. One of these, Lys514, is a residue close to Arg518. The finding that the replacement of Arg518 by a residue with a much shorter and uncharged side-chain results in a dramatic decrease of the affinity for fructose 6-phosphate and fructose 1-phosphate, confirms our previous observations and indicates that Arg518 might also directly participate in the binding of the two fructose-phosphates. This mutation also causes GKR inhibition of glucokinase to be much less dependent on fructose 6-phosphate. This, together with the fact that the Arg518Gln mutation almost abolishes the affinity of GKR for fructose 1-phosphate, makes the mutant GKR a more powerful inhibitor of glucokinase. However, this strengthening of the inhibition is largely attenuated by a decrease in stability, which results in lower levels of expression in mammalian cells. Therefore, the overall result of the Arg518Gln mutation is probably not very different from a null mutation. If this is the case, the same kind of reasoning could then apply for this mutation as for the premature stop codon. Accordingly, analysis of the family bearing this mutation indicates that it is also not linked to obesity.

This conclusion is in agreement with the finding that the GKR knock-out mice models, whether heterozygous or homozygous, have a normal weight [41, 42]. Moreover, when challenged with a high-sucrose/high-fat diet the knock-out mice and the normal mice gained body weight at a similar rate [41]. The description of the phenotype of these mice shows, nevertheless, that the expression of only one copy of the GKR gene destabilises glucokinase in the liver and reduces its activity by 16 to 38%. The decrease or ablation of liver glucokinase was mimicked in another mouse model [43] and again these animals did not suffer from obesity. Interestingly, the blood glucose level of the subjects with mutation Arg227Stop and Arg518Gln was not abnormal, indicating that the potential change in liver glucokinase induced by these mutations is not sufficient to perturb glucose homeostasis.

In conclusion, mutations in the GKR gene can be excluded as a frequent cause contributing to obesity in the French Caucasian population. Some rare variants with consequential effect on the protein function exist in this population, but as such they most probably have no phenotypic effect related to energy and glucose homeostasis, at least in the heterozygous state.

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