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B.R. Barnes, S. Marklund, T.L. Steiler, M. Walter, G. Hjalms, et al.. The 5'-AMP-activated protein kinase gamma 3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle.. *Journal of Biological Chemistry*, 2004, 279, pp.38441-38447. hal-01211883

HAL Id: hal-01211883

<https://hal.science/hal-01211883>

Submitted on 31 May 2020

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The 5'-AMP-activated Protein Kinase $\gamma 3$ Isoform Has a Key Role in Carbohydrate and Lipid Metabolism in Glycolytic Skeletal Muscle*

Received for publication, May 18, 2004, and in revised form, June 23, 2004
Published, JBC Papers in Press, July 6, 2004, DOI 10.1074/jbc.M405533200

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5'-AMP-activated protein kinase (AMPK) is a metabolic stress sensor present in all eukaryotes. A dominant missense mutation (R225Q) in pig *PRKAG3*, encoding the muscle-specific $\gamma 3$ isoform, causes a marked increase in glycogen content. To determine the functional role of the AMPK $\gamma 3$ isoform, we generated transgenic mice with skeletal muscle-specific expression of wild type or mutant (225Q) mouse $\gamma 3$ as well as *Prkag3* knock-out mice. Glycogen resynthesis after exercise was impaired in AMPK $\gamma 3$ knock-out mice and markedly enhanced in transgenic mutant mice. An AMPK activator failed to increase skeletal muscle glucose uptake in AMPK $\gamma 3$ knock-out mice, whereas contraction effects were preserved. When placed on a high fat diet, transgenic mutant mice but not knock-out mice were protected against excessive triglyceride accumulation and insulin resistance in skeletal muscle. Transfection experiments reveal the R225Q mutation is associated with higher basal AMPK activity and diminished AMP dependence. Our results validate the muscle-specific AMPK $\gamma 3$ isoform as a therapeutic target for prevention and treatment of insulin resistance.

AMPK¹ is a heterotrimeric serine/threonine protein kinase composed of a catalytic α subunit and non-catalytic β and γ subunits (1, 2). The mammalian genome contains seven AMPK

genes encoding two α , two β , and three γ isoforms. AMPK signaling is elicited by cellular stresses that deplete ATP (and consequently elevate AMP) by either inhibiting ATP production (e.g. hypoxia) or accelerating ATP consumption (e.g. muscle contraction). AMPK is activated allosterically by AMP and through phosphorylation of Thr¹⁷² in the α subunit by an upstream AMPK kinase, the tumor-suppressor protein kinase LKB1 (3, 4). AMPK is likely to be important for diverse functions in many cell types, but particular interest has been focused on elucidating the role of AMPK in the regulation of lipid and carbohydrate metabolism in skeletal muscle (5–10). AMPK activity has been correlated with an increase in glucose uptake and fatty acid oxidation and an inhibition of glycogen synthase activity and fatty acid synthesis. Exercise, as well as skeletal muscle contractions *in vitro*, leads to AMPK activation. Pharmacological activation of AMPK also can be achieved using 5-aminoimidazole-4-carboxamide-1- β -D-ribose nucleoside (AICAR). Once taken up by the cell, AICAR is phosphorylated to 5-aminoimidazole-4-carboxamide riboside monophosphate (ZMP) and mimics effects of AMP on AMPK (1, 2). AMPK function is closely related to glycogen storage. AMPK phosphorylates glycogen synthase *in vitro* (11) and co-immunoprecipitates with glycogen synthase and glycogen phosphorylase from skeletal muscle (12). Mutations of the $\gamma 3$ or $\gamma 2$ subunit, respectively, affect glycogen storage in pigs (13, 14) or glycogen storage associated with cardiac abnormalities in humans (15). The recent identification of a glycogen-binding domain in the AMPK $\beta 1$ subunit provides a molecular relationship between AMPK and glycogen (16, 17). The formation of heterotrimers appears to be rather promiscuous, and the different subunits ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, $\gamma 1$, $\gamma 2$, and $\gamma 3$) can form a maximum of 12 different AMPK heterotrimers. The functional diversification of the different isoforms is largely unknown.

The dominant Rendement Napole (RN) phenotype identified in Hampshire pigs is associated with a single missense mutation (R225Q) in *PRKAG3*, encoding the muscle-specific AMPK $\gamma 3$ isoform (13). RN pigs have a ~70% increase in glycogen content in glycolytic skeletal muscle, whereas liver and heart glycogen content is unchanged (18, 19). The mutation has a large impact on meat characteristics and leads to a low pH because of the anaerobic glycogen degradation occurring post-mortem. A second mutation (V224I) identified in pigs at the neighboring amino acid residue is associated with an opposite

* This work was supported by grants from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, the Swedish Medical Research Council, the Swedish Diabetes Association, the Foundation for Scientific Studies of Diabetology, and the Novo-Nordisk Research Foundation. 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.

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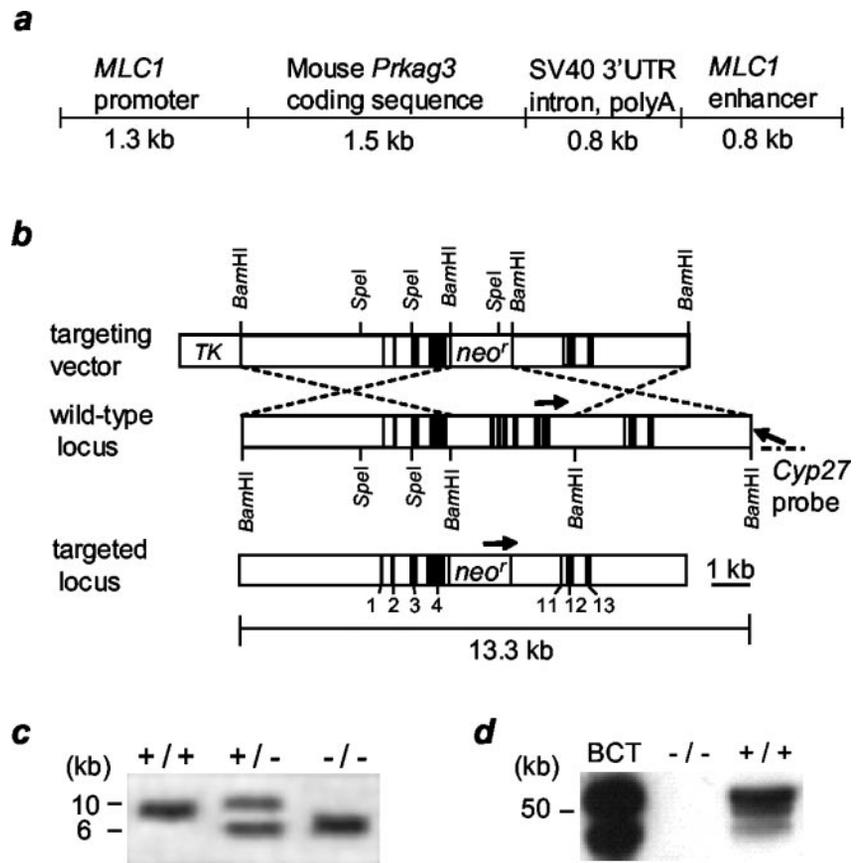
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^l The abbreviations used are: AMPK, 5'-AMP-activated protein kinase; AICAR, 5-aminoimidazole-4-carboxamide-1- β -D-ribose nucleoside;

RN, Rendement Napole; KHB, Krebs-Henseleit bicarbonate buffer; EDL, extensor digitorum longus; ACC, acetyl-CoA carboxylase.

FIG. 1. Overview of *Tg-Prkag3* transgene and knock-out targeting constructs. *Tg-Prkag3* transgene constructs and a knock-out targeting construct including the neomycin resistance (*neo^r*) and thymidine kinase (*TK*) genes are shown in *a* and *b*, respectively. The wild type locus and the targeted locus are shown with exons indicated by black boxes and numbers. BamHI and SpeI sites, primers used for long range PCR, and the location of the *Cyp27* locus are indicated by arrows. UTR, untranslated region. *c*, Southern blot of SpeI-digested genomic DNA from wild type (+/+), heterozygous (+/-), and knock-out homozygote (-/-) mice, probed with the flanking *Cyp27* probe. *d*, Western blot analysis of gastrocnemius muscle extracts from *Prkag3^{-/-}* and *Prkag3^{+/+}* mice. The apparent double band for AMPK $\gamma 3$ was typically observed after separation by SDS-PAGE and may relate to a protease-sensitive site within the protein. Corresponding bacterially expressed His-tagged proteins (*BCT*) were included as a positive control.



effect, low glycogen and high pH, compared with the *RN* allele (14). We have found that $\gamma 3$ is the predominant AMPK γ isoform in glycolytic (white, fast-twitch type II) muscle, whereas it is expressed at very low levels in oxidative (red, slow-twitch type I) muscle and is undetectable in brain, liver, or white adipose tissue (20). Furthermore, $\gamma 3$ primarily forms heterotrimers with $\alpha 2$ and $\beta 2$ isoforms in glycolytic skeletal muscle. Here we report the characterization of the metabolic consequences of genetic modification of AMPK $\gamma 3$ expression in skeletal muscle.

MATERIALS AND METHODS

Animal Care—Mice were maintained in a temperature- and light-controlled environment and were cared for in accordance with regulations for the protection of laboratory animals. The regional animal ethical committee approved all experimental procedures. Animals had free access to water and standard rodent chow. In some experiments, female mice were placed on either a standard chow or a high fat diet (21) from 4 to 16 weeks of age.

Generation of Transgenic Mice—The complete coding sequence of mouse *Prkag3* was amplified by reverse transcriptase-PCR using skeletal muscle mRNA (Clontech). The forward (5'-CACCATGGAGCCGAGCTGGAGCA) and reverse (5'-GTCTCAGGCGCTGAGGGC-ATC) primers included the translation start and stop codons (in bold), respectively. The forward primer also included a Kozak element (CACC, underlined above) (22) in front of the start codon to facilitate initiation of translation. The reverse transcriptase-PCR product (~1.5 kb) was ligated into the pCRII TA TOPO cloning vector (Invitrogen). A clone with this consensus sequence (100% identity to *Prkag3*) was used for the transgene constructs. The R225Q mutation (13) was introduced by *in vitro* mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene) changing codon 225 from arginine to glutamine. EcoRI fragments containing the wild type or the mutant (225Q) form were ligated into the pMLC vector (23) along with flanking sequences for the myosin light chain 1 (*MLC1*) promoter and enhancer and the SV40 3'-untranslated region (Fig. 1*a*). The constructs were cut from the plasmid and microinjected into mouse oocytes (CBA \times C57Bl/6J).

The founder mice were tested for transgenesis by PCR analysis using a forward primer in *Prkag3* exon 12 (5'-GCTGCCAGCAAACCTAC-

AAC) and a reverse primer in SV40 3'-untranslated region. This amplicon spans the SV40 3'-untranslated region intron (66 bp) and thereby allowed for confirmation of the transgene both in genomic DNA (amplicon size = 453 bp) and in cDNA (amplicon size = 387 bp). Genomic DNA prepared from mouse tails was used for PCR.

Generation of Knock-out Mice—*Prkag3* knock-out mice were generated through traditional gene-targeting techniques. Briefly, exons 1–4 and exons 11–13 of *Prkag3* were cloned into the pKOV923 selection plasmid (Stratagene) with a neomycin resistance (*neo^r*) gene inserted. The predicted result was a *Prkag3* transcript with exons 1–4 joined with exons 11–13, including a frameshift after residue 211 and a premature stop codon at residue 235, skipping most of the 489 amino acids encoded by the wild type transcript (Fig. 1*b*). The construct was linearized using NotI and used for electroporation of embryonic stem cells. Knock-out recombinant embryonic stem cells were injected into blastocysts. Screening for knock-out recombinant embryonic stem cells was performed using Southern analysis, with SpeI digestions and a 1034-bp cDNA probe, representing the mitochondrial vitamin D (3) 25-hydroxylase (*Cyp27*) gene (Fig. 1*b*). The probe was amplified by reverse transcriptase-PCR from mouse skeletal muscle mRNA. Long range PCR (MasterAmp high fidelity long PCR kit, Epicenter Technologies) was used to screen for knock-out recombinant embryonic stem cells and identification of heterozygous (*Prkag3^{+/-}*) and homozygous (*Prkag3^{-/-}*) carriers of the knock-out recombinant allele, respectively. Founder mice were back-crossed to C57Bl/6J mice for three generations. In all experiments, knock-out homozygote mice were compared with homozygous wild type littermates.

Relative Quantification of mRNA—Quantification of mRNA representing different isoforms of AMPK γ subunits from adult mouse tissues was performed using reverse transcription and real time PCR. Relative quantities of mRNA were calculated for duplicate tissue samples from 1–2 mice and normalized for *Actb* (β -actin).

Cell Culture and Transfections—Briefly, cDNA encoding the $\gamma 3$ subunit of AMPK was inserted into cloning vector pDONR201 included in the Gateway cloning system (Invitrogen) per the manufacturer's instructions. Site-directed mutagenesis was used to create $\gamma 3$ V224I and $\gamma 3$ R225Q cDNA constructs, which were cloned into Gateway cloning system pDEST26 (Invitrogen) for subsequent expression in mammalian cell culture.

Cultured COS7 cells were transiently transfected with cDNA encod-

TABLE I

Relative mRNA expression levels from endogenous *Prkg1* and *Prkg3* and transgenic *Prkg3* in mice as measured by real time PCRResults are relative to the calibrator value indicated in bold. The results are based on duplicate samples from 1–3 individuals/(tissue and line of mice). Average values \pm S.E. are given in cases where 2–3 samples were tested.

Tissue	Mouse line	<i>Prkg1</i> endogenous	<i>Prkg3</i>	
			Endogenous	Transgenic
Liver	WT ^a	2.4 \pm 0.9	0.0	
	AMPK $\gamma 3^{wt}$	1.7	0.0	0.0
	AMPK $\gamma 3^{225Q}$	2.7 \pm 1.3	0.0	0.0
Heart	WT	22.9 \pm 9.3	1.2 \pm 0.4	
	AMPK $\gamma 3^{wt}$	12.5	0.1	0.0
	AMPK $\gamma 3^{225Q}$	12.9 \pm 10.9	0.3 \pm 0.1	0.0
Extensor digitorum longus ^b	WT	19.9 \pm 14.9	24.2 \pm 3.0	
	AMPK $\gamma 3^{wt}$	23.3 \pm 3.5	18.3 \pm 4.2	1646 \pm 492
	AMPK $\gamma 3^{225Q}$	18.7	14.1	73.2
Gastrocnemius ^b	WT	30.9 \pm 0.8	100 \pm 25.3	
	AMPK $\gamma 3^{wt}$	26.3 \pm 8.5	62.1 \pm 13.2	659 \pm 222
	AMPK $\gamma 3^{225Q}$	19.4 \pm 8.0	11.1 \pm 2.4	99.5 \pm 28.3
	AMPK $\gamma 3^{-/-}$	9.5	3.8	
Quadriceps ^b	WT	45.7 \pm 3.1	41.3 \pm 3.2	
	AMPK $\gamma 3^{wt}$	27.5	26.8	167.2
	AMPK $\gamma 3^{225Q}$	35.0	12.9	182.8
Soleus ^c	WT	33.4 \pm 8.7	3.0 \pm 0.8	
	AMPK $\gamma 3^{wt}$	30.8 \pm 0.6	2.6 \pm 0.8	11.1 \pm 6.9
	AMPK $\gamma 3^{225Q}$	28.7 \pm 5.2	2.7 \pm 0.5	6.5 \pm 4.5
Diaphragm ^c	WT	7.0 \pm 4.0	1.3 \pm 0.6	
	AMPK $\gamma 3^{wt}$	12.8	2.5	5.0
	AMPK $\gamma 3^{225Q}$	10.3 \pm 8.0	1.3 \pm 0.8	0.9 \pm 0.9
Brain	WT	1.5 \pm 0.0	0.0	
	AMPK $\gamma 3^{wt}$	2.2 \pm 0.3	0.0	0.0
	AMPK $\gamma 3^{225Q}$	1.7	0.0	0.0
White adipose tissue	WT	1.3 \pm 0.4	0.0	
	AMPK $\gamma 3^{wt}$	0.9	0.0	0.0
	AMPK $\gamma 3^{225Q}$	1.2	0.0	0.0

^a WT, wild type.^b Muscle containing primarily glycolytic muscle fibers.^c Muscle containing primarily oxidative muscle fibers.

ing $\alpha 2$, $\beta 2$, and $\gamma 3$ wild type, $\gamma 3$ V224I, or $\gamma 3$ R225Q. Post-transfection, cells were lysed, insoluble material was removed, and lysates were exposed to protein G-Sepharose-bound monoclonal antibody. After incubation, $\alpha 2$ containing immune complexes were harvested, washed, and halved for subsequent activity assays and immunoblotting.

AMPK Activity Assay—AMPK activity was measured by phosphate incorporation of the ADR1 (222–234)Pro²²⁹ peptide substrate, LKKLTLRPSFSAQ (24). AMPK immunocomplexes were assayed for 10 min, reactions were spotted on phosphocellulose paper (P81), and radioactivity was assessed by liquid scintillation analyzer. AMPK activities were calculated as pmol of phosphate incorporated into the ADR1 peptide/min in the presence of equal amounts of the heterotrimer.

Western Blot Analysis—Quantitative analysis of the expression of different AMPK subunits was performed as described previously (20). Skeletal muscle protein lysate was separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and probed with primary AMPK isoform- or phospho-specific antibodies and secondary horseradish peroxidase-conjugated antibodies.

Glycogen and Triglyceride Analyses—Mice were studied under fed or fasted conditions or after swim exercise, as described previously (25). Fasted mice swam for four 30-min intervals separated by 5-min rest periods. After the last swim interval, mice were studied immediately or dried and returned to cages for 2.5 h (recovery). At the onset of the recovery period, mice received an intraperitoneal glucose injection (0.5 mg/g of body mass) and were subsequently given free access to chow and water. Gastrocnemius muscles were removed from anesthetized mice (Avertin; 2,2,2-tribromoethanol 99+ and tertiary amyl alcohol, 15 μ l/g of body mass), cleaned of fat and blood, and quickly frozen in liquid nitrogen. Glycogen content was determined fluorometrically on HCl extracts as described previously (26). Triglyceride content was determined with a triglycerides/glycerol blanked kit (Roche Applied Science) using SeronormTM lipid (SERO) as a standard.

Glucose Tolerance Test—Glucose (2 g/kg of body mass) was administered to fasted mice by intraperitoneal injection. Blood samples were obtained via the tail vein prior to and 15, 30, 60, and 120 min following glucose injection for measurement of glucose concentration (One Touch Basic glucose meter; Lifescan).

Skeletal Muscle Incubation Procedure—Incubation medium was prepared from a stock solution of Krebs-Henseleit bicarbonate buffer

(KHB) supplemented with 5 mmol/liter HEPES and 0.1% bovine serum albumin (RIA grade) and continuously gassed with 95% O₂, 5% CO₂. Mice were anesthetized, and extensor digitorum longus (EDL) muscles were isolated and preincubated at 30 °C for 30 (glucose uptake) or 60 (for AMPK and acetyl-CoA carboxylase (ACC) phosphorylation) min in KHB containing 5 mmol/liter glucose and 15 mmol/liter mannitol in the absence or presence of insulin (12 nmol/liter) or AICAR (2 mmol/liter). Some muscles were subjected to 10 min of *in vitro* electrical stimulation, as described previously (27), before (glucose uptake) or during the final 10 min (for AMPK and ACC phosphorylation) of the incubation.

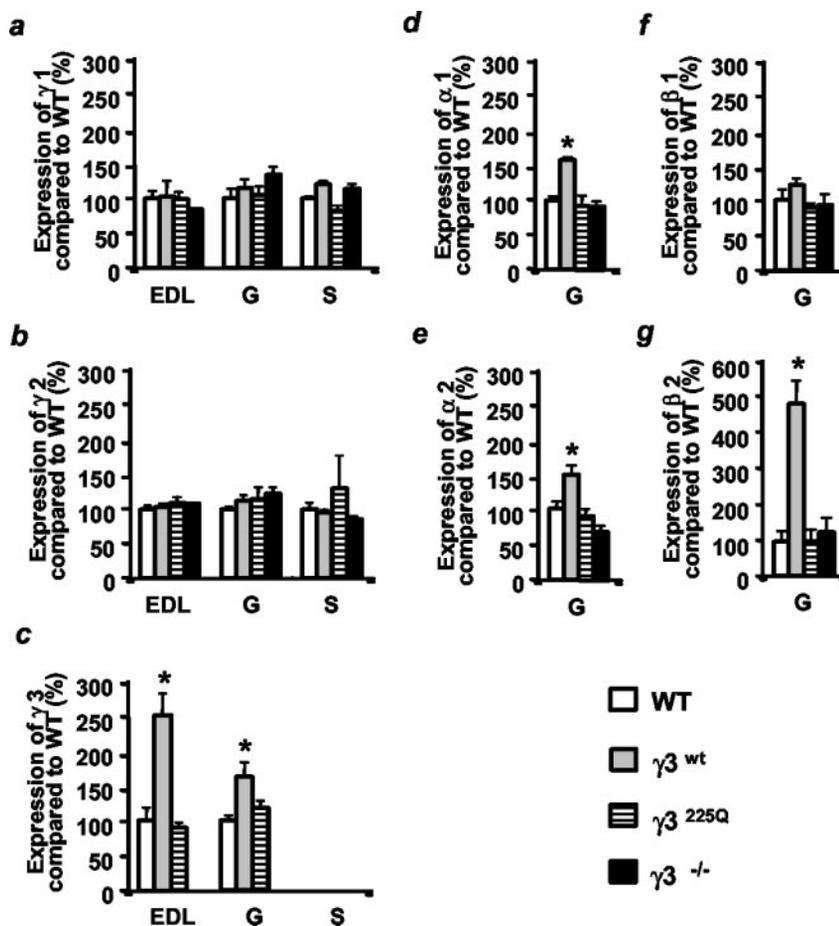
Metabolic Assays—2-Deoxyglucose uptake was assessed in EDL muscles as described previously (26). Results are expressed as μ mol/ml of intracellular water/h (26). Oleate oxidation was assessed in EDL muscles as described by Young *et al.* (28) with minor modifications. Muscles were preincubated in the presence of insulin (60 nmol/liter) without or with AICAR (2 mmol/liter) for 20 min in KHB containing 5 mmol/liter HEPES, 3.5% fatty acid-free bovine serum albumin, and 10 mmol/liter glucose. Thereafter, muscles were incubated in 1 ml of identical medium containing 0.3 mmol/liter [1-¹⁴C]oleate (0.4 μ Ci/ml) for 60 min. The medium was acidified by 0.5 ml of 15% Δ^1 -pyrroline-5-carboxylic acid, and liberated CO₂ was collected in center wells containing 0.2 ml of Protosol (PerkinElmer Life Sciences) for 60 min. Center wells were removed for scintillation counting. Results were expressed as nmol of oxidized oleate/g of wet mass/h.

Statistical Analyses—Differences between two groups were determined by an analysis of variance with multiple comparisons. Differences between more than two groups were determined by one-way analysis of variation followed by Fisher's least significant difference *post hoc* analysis. Significance was accepted at $p < 0.05$.

RESULTS AND DISCUSSION

Three novel mouse models were used to genetically dissect the functional role of the AMPK $\gamma 3$ isoform in skeletal muscle. AMPK $\gamma 3$ -transgenic (*Tg-Prkg3^{wt}* and *Tg-Prkg3^{225Q}*) and *Prkg3^{-/-}* mice had normal growth rates (data not shown). The expression of *Prkg3* wild type (*Tg-Prkg3^{wt}*) or an R225Q mutant form (*Tg-Prkg3^{225Q}*) transgene was restricted to skeletal muscles containing a high proportion of glycolytic fibers,

FIG. 2. AMPK isoform expression in skeletal muscle. Shown are (a) $\gamma 1$, (b) $\gamma 2$, (c) $\gamma 3$, (d) $\alpha 1$, (e) $\alpha 2$, (f) $\beta 1$, and (g) $\beta 2$ subunits in wild type (*wt*, open bars), transgenic wild type ($\gamma 3^{wt}$, gray bars), transgenic mutant ($\gamma 3^{225Q}$, bars marked with horizontal lines), and knock-out ($\gamma 3^{-/-}$, filled bars) mice. Results are based on quantitative Western blot analysis of muscle extracts (G, gastrocnemius; S, soleus) using isoform-specific antibodies. Only comparisons of the relative expression of the same subunit between lines of mice are valid. Relative expression of a given isoform in wild type was used as a calibrator and given the value 100. An asterisk indicates that $p < 0.05$ versus respective wild type.



consistent with the expression profile of the endogenous *Prkag3* transcript (Table I). We found a marked overexpression of the wild type transgene in EDL, gastrocnemius, and quadriceps muscle (~16.5-, 6.6-, and 1.7-fold, respectively), but no or only a moderate overexpression of the mutant transgene (~0.7-, 1.0-, and 1.8-fold, respectively). Positional effects or number of integrated copies most likely explain the difference in expression level between the two transgenic models. Levels of endogenous *Prkag3* transcript in *Tg-Prkag3^{225Q}* mice tended to be decreased.

The amount of expressed $\gamma 3$ protein, as well as α , β , or the other γ subunits, was unchanged in *Tg-Prkag3^{225Q}* mice (Fig. 2). Thus, AMPK expression in *Tg-Prkag3^{225Q}* mice resembles the expression pattern in wild type mice, both in regard to tissue distribution and protein expression. The mutant form (225Q) presumably replaced endogenous $\gamma 3$, based on the relative mRNA expression. However, endogenous and exogenous forms, as assessed by Western blot, were indistinguishable because they differed by a single amino acid substitution. An absolute quantification of the relative expression of the mutant and wild type protein is not crucial for the interpretation of our results because the R225Q mutation is fully dominant. Moreover, there is no significant difference in glycogen content between pigs expressing 50 or 100% of the mutant form.² Overexpression of the wild type transgene led to an increase in the amount of $\gamma 3$ protein and a concomitant increase in $\alpha 1$, $\alpha 2$, and $\beta 2$ subunits (Fig. 2). Thus, the total amount of AMPK heterotrimers in glycolytic muscles was increased in *Tg-Prkag3^{wt}* mice.

Southern and Western blot analysis confirmed the successful disruption of *Prkag3* and concomitant complete absence of $\gamma 3$ expression in skeletal muscle in *Prkag3* knock-out (*Prkag3^{-/-}*)

mice (Fig. 1, c and d). The homozygous knock-out animals were fully viable, and a standard pathological examination revealed no obvious phenotypic consequences of the $\gamma 3$ disruption. Real time PCR analysis of mRNA from skeletal muscle expectedly revealed a low abundance of *Prkag3* transcripts, as the PCR primers were designed against a part of the 3'-region that was not deleted by the gene-targeting event (Fig. 1b and Table I). The low abundance of this aberrant transcript likely reflects degradation by the nonsense-mediated mRNA decay pathway (29). Western blot analysis did not reveal any compensatory increase in $\gamma 1$ and $\gamma 2$ isoform expression in skeletal muscle (Fig. 2), indicating that these isoforms do not compete with $\gamma 3$ for the same pool of α - β chains or do not form AMPK heterotrimers in the same cell or cellular compartment.

Glycogen content in the glycolytic portion of the gastrocnemius muscle was 2-fold higher in *Tg-Prkag3^{225Q}* mice compared with wild type mice under both fed and fasted conditions, whereas glycogen content was unaltered in *Tg-Prkag3^{wt}* or *Prkag3^{-/-}* mice (Fig. 3a). The results provide definitive evidence that R225Q is the causative mutation for the RN phenotype in pigs (13) because the phenotype is replicated in mice by introducing this single missense mutation. Furthermore, this mutation alters the biochemical regulation of AMPK, as the increase in AMPK expression in the *Tg-Prkag3^{wt}* mice failed to cause a glycogen phenotype.

Glycogen content after swimming exercise was similar between wild type and all genetically modified mice (Fig. 3a). Thus, the R225Q mutation does not impair glycogen utilization during exercise. Because postexercise glycogen levels were appropriately depleted in *Prkag3^{-/-}* mice, our data also suggests that AMPK $\gamma 3$ is not required for glycogen degradation. Glycogen content 2.5 h after exercise was significantly higher in *Tg-Prkag3^{225Q}* mice compared with wild type mice (Fig. 3a). A

² L. Andersson, unpublished observation.

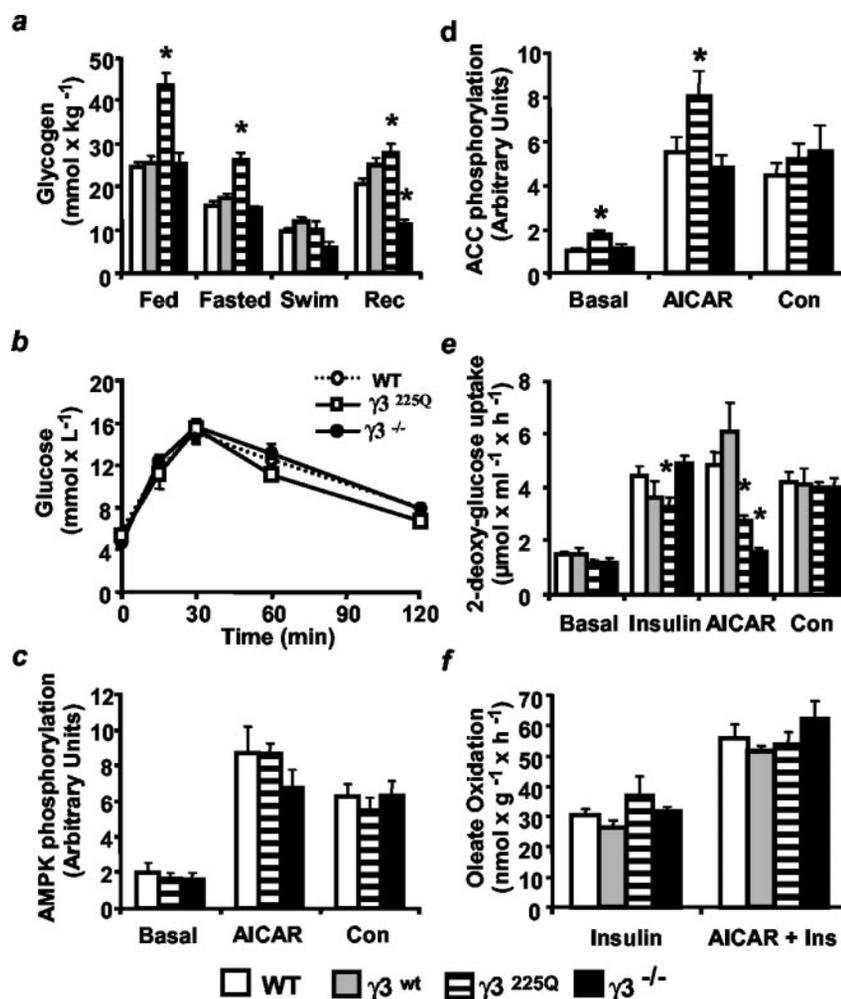


FIG. 3. Genetic modification of AMPK $\gamma 3$ dramatically affects skeletal muscle metabolism. *a*, glycogen content in gastrocnemius tissue from mice under *Fed*, *Fasted*, *Swim* (2 h), and *Rec* (2.5-h recovery after swim) conditions ($n = 5-30$). Fasted mice were studied in *b-f*. *b*, glucose tolerance ($n = 10-15$). *c-f*, isolated EDL muscle was studied under basal conditions or after stimulation with insulin (*Ins*; 12 nmol/liter), AICAR (2 mmol/liter), or electrically induced contraction (*Con*; 20 Hz for 10 min). *c*, AMPK phosphorylation ($n = 6-10$). *d*, ACC phosphorylation ($n = 6-12$). *e*, 2-deoxyglucose uptake ($n = 4-20$). *f*, oleate oxidation ($n = 4-18$). An asterisk indicates that $p < 0.05$ versus respective basal. WT, wild type.

similar tendency (N. S) for increased glycogen content after exercise was also noted in *Tg-Prkag3^{wt}* mice. In contrast, glycogen content was significantly lower in *Prkag3^{-/-}* versus wild type mice 2.5 h after exercise, demonstrating that $\gamma 3$ is important for glycogen resynthesis.

Fasted insulin and glucose levels (data not shown) and glucose tolerance (Fig. 3*b*) were normal in transgenic and *Prkag3^{-/-}* mice. Thus, despite a very distinct phenotype for skeletal muscle glycogen content, blood glucose homeostasis was normal in *Tg-Prkag3^{225Q}* mice, consistent with the phenotype noted in pigs carrying the R225Q mutation.³

AMPK phosphorylation under basal conditions or after activation with AICAR or contraction was similar between genotypes (Fig. 3*c*). Phosphorylation of the AMPK downstream target ACC was elevated under basal conditions and after AICAR stimulation in *TgPrkag3^{225Q}* mice (Fig. 3*d*). This was unexpected because our transfection experiments (see below) revealed that the R225Q mutant $\gamma 3$ isoform is AMP-independent and thus would be predicted to be AICAR-insensitive. However, the elevated ACC phosphorylation may be an indirect effect of the R225Q mutant $\gamma 3$ isoform caused by an altered metabolic state of the cell. In fact, the $\gamma 3$ isoform may not mediate ACC phosphorylation, consistent with unaltered ACC phosphorylation in *Prkag3^{-/-}* mice (Fig. 3*d*).

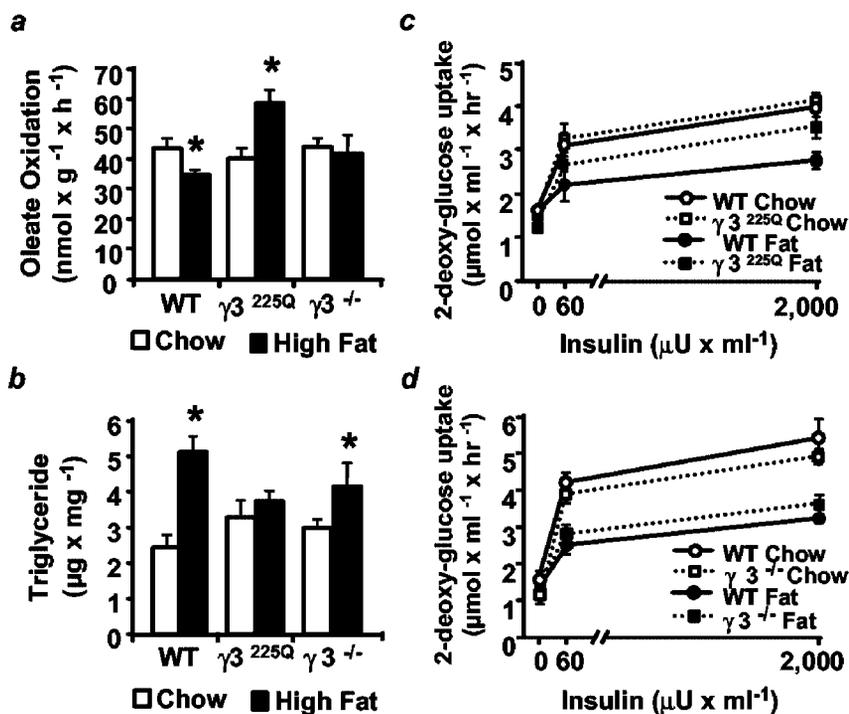
Glucose transport in isolated EDL muscle from fasted mice was determined in response to insulin, in response to AICAR, or after electrically stimulated contractions (Fig. 3*e*). Basal

glucose transport was similar between genotypes. Insulin-stimulated glucose transport was normal in *Tg-Prkag3^{wt}* and *Prkag3^{-/-}* mice but was significantly reduced in *Tg-Prkag3^{225Q}* mice. The reduction in insulin-stimulated glucose transport was not observed in fed mice (data not shown). AICAR-induced glucose transport was normal in *Tg-Prkag3^{wt}*, but significantly reduced (~50%) in *Tg-Prkag3^{225Q}*, compared with wild type fasted (Fig. 3*e*) and fed (data not shown) mice. Thus, the mutation in $\gamma 3$ may occur at a site that is directly involved with the interaction with both AMP and AICAR, rendering a mutant form that is partially resistant to AICAR. This interpretation is supported by evidence that this region of the γ subunit directly binds AMP and that the presence of this mutation at the corresponding site in the $\gamma 1$ (R70Q) or $\gamma 2$ (R302Q) subunit impairs AMP and ATP binding (30, 31). However, we cannot exclude a partial inhibition because of excessive glycogen content (32, 33). Interestingly, the AICAR effect on glucose uptake in EDL muscle was completely abolished in *Prkag3^{-/-}* mice (Fig. 3*e*). Thus, AMPK complexes containing the $\gamma 3$ subunit are required for AICAR-induced glucose transport in skeletal muscle, and other γ isoforms fail to compensate for the loss of $\gamma 3$ function. This result is consistent with the reduced glycogen resynthesis *in vivo* after exercise in *Prkag3^{-/-}* mice (Fig. 3*a*).

In contrast to the results for AICAR, *in vitro* contraction of isolated EDL muscle led to a similar increase in glucose uptake in all genotypes in fasted (Fig. 3*e*) or fed mice (data not shown). Similarly, AICAR- but not contraction-induced glucose uptake was abolished in AMPK $\alpha 2$, but not in $\alpha 1$ knock-out mice (34). Because the $\gamma 3$ subunit primarily forms heterotrimers with $\alpha 2$ (20), disruption of either $\alpha 2$ or $\gamma 3$ should confer a similar

³ B. Essén-Gustavsson, M. Jensen-Waern, R. Jonasson, and L. Andersson, unpublished observation.

FIG. 4. $\gamma 3^{225Q}$ prevents dietary induced insulin resistance through increased fat oxidation. Wild type (WT), AMPK $\gamma 3$ mutant-overexpressing ($\gamma 3^{225Q}$), and knockout ($\gamma 3^{-/-}$) mice were provided chow or a high fat diet for 12 weeks. Fed mice were studied in *a-d*. *a*, oleate oxidation in EDL muscle ($n = 4-18$). *b*, triglyceride content in gastrocnemius muscle ($n = 7-11$). *c* and *d*, 2-deoxyglucose uptake in EDL muscle from (*c*) wild type versus $\gamma 3^{225Q}$ mice ($n = 6-8$) and (*d*) wild type versus $\gamma 3^{-/-}$ mice ($n = 4-5$). An asterisk indicates that $p < 0.05$ chow versus high fat diet.



glucose transport defect in skeletal muscle. AICAR-induced glucose uptake was also abolished in kinase-dead AMPK $\alpha 2$ -transgenic mice (35). In contrast to the $\alpha 2$ AMPK knock-out and the *Prkag3*^{-/-} mice, contraction-mediated glucose transport was significantly blunted (30%) in kinase-dead AMPK $\alpha 2$ -transgenic mice, possibly because of contraction-induced hypoxia, as these mice have an impaired hypoxia response. Collectively, these results challenge the hypothesis that contraction increases glucose transport through an AMPK-mediated mechanism. In contrast, activation of AMPK is directly linked to AICAR-stimulated glucose transport. Although AICAR and contraction both increase AMPK activity, the AMPK response to *in vitro* contraction may be inconsequential for activation of glucose transport. Although $\gamma 3$ -containing AMPK complexes are required for AICAR-mediated glucose uptake, they appear to be dispensable for AICAR-mediated fatty acid oxidation in chow-fed mice. AICAR-mediated fatty acid oxidation was similar between genotypes (Fig. 3*f*), consistent with the observed normal level of ACC phosphorylation in the *Prkag3*^{-/-} mice (Fig. 3*d*).

AMPK has been identified as a molecular target for pharmacological intervention to treat insulin resistance and type II diabetes mellitus. However, genetic validation of this target is lacking. We challenged wild type, *Tg-Prkag3*^{225Q}, and *Prkag3*^{-/-} mice with a high fat diet for 12 weeks and evaluated metabolic responses. Muscle glycogen content was unaffected by the high fat diet (data not shown). However, triglyceride content was increased (Fig. 4*b*), and insulin action on glucose transport was impaired in wild type and *Prkag3*^{-/-} mice (Fig. 4, *c* and *d*). In contrast, *Tg-Prkag3*^{225Q} mice were protected against triglyceride accumulation (Fig. 4*b*) and insulin resistance (Fig. 4*c*), presumably because of increased fat oxidation (Fig. 4*a*) in skeletal muscle. This phenotype closely resembles the original phenotype described for mutant pigs (13). The high frequency of the *RN*⁻ mutation (*PRKAG3*^{225Q}) in Hampshire pigs was likely caused by the strong selection for lean meat content in commercial pig populations, as pigs carrying this mutation are leaner (more muscle, less fat) than wild type pigs. Mutant pigs also have a higher oxidative capacity, as measured

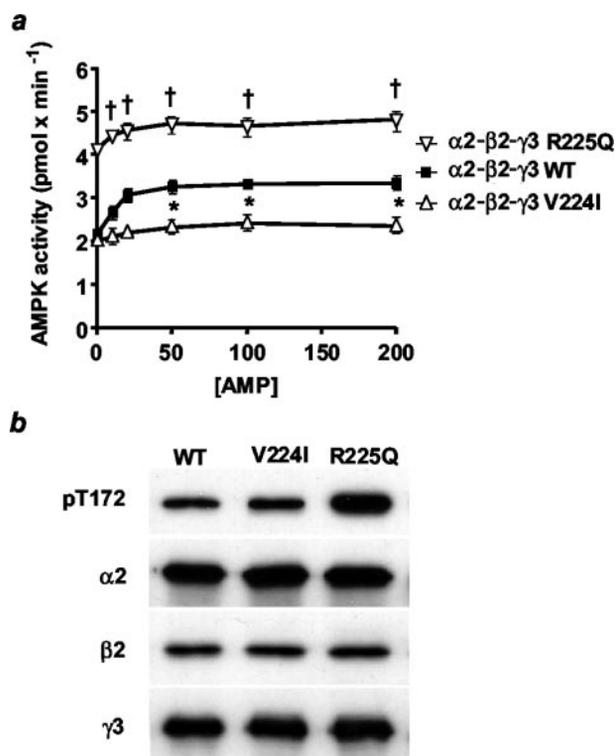


FIG. 5. AMPK activation in COS cells expressing trimers containing $\alpha 2$ - $\beta 2$ - $\gamma 3$ wild type or mutant $\gamma 3$ (R225Q or V224I). *a*, AMPK activity in the presence of AMP (0–200 μM). Values are pmol of phosphate incorporated \times min⁻¹ ($n = 4$). *b*, representative Western blot analysis using antiphospho-Thr¹⁷², anti- $\alpha 2$, anti- β , and anti- $\gamma 3$ antibodies. An asterisk indicates that $p < 0.05$, and a dagger indicates that $p < 0.01$. WT, wild type.

by an increase in activity of citrate synthase and β -hydroxyacyl-coenzyme A dehydrogenase (36, 37).

The relationship between the R225Q mutation and increased oxidative capacity in both mutant pigs and transgenic mice prompted us to investigate whether this was associated with an

altered muscle fiber type composition. Real time PCR analysis of *Atp1b1* and *Atpb2* (20), markers of glycolytic and oxidative skeletal muscle fibers (38), revealed a similar ratio of *Atp1b1* and *Atpb2* expression in gastrocnemius and EDL muscle from *Tg-Prkg3^{225Q}* and *Prkg3^{-/-}* mice (data not shown). Furthermore, myoglobin protein expression in *Tg-Prkg3^{225Q}* and *Prkg3^{-/-}* did not differ from wild type mice (data not shown). Thus, R225Q alters skeletal muscle oxidative capacity without altering fiber type composition.

AMPK activity and phosphorylation of Thr¹⁷² were determined in COS cells transfected with AMPK trimers containing $\alpha 2$, $\beta 2$, and either wild type $\gamma 3$ or mutant $\gamma 3$ (R225Q or V224I). AMPK activity and phosphorylation on Thr¹⁷² in the absence of AMP were elevated in cells transfected with $\alpha 2$ - $\beta 2$ - $\gamma 3$ R225Q and unchanged in cells transfected with $\alpha 2$ - $\beta 2$ - $\gamma 3$ V224I. Both mutations resulted in diminished AMP dependence on AMPK (Fig. 5). The ranking of basal AMPK activity in the three genotypes is consistent with the *in vivo* effects of the corresponding pig mutations, as the R225Q and V224I mutants are associated with increased and decreased muscle glycogen content, respectively (13, 14).

CONCLUSION

We provide definitive evidence that *PRKAG3* R225Q is the causative mutation for the elevated glycogen content in skeletal muscle from RN pigs. *Tg-Prkg3^{225Q}* mice have elevated glycogen levels and have increased glycogen resynthesis after exercise. Our *in vitro* studies reveal that AMPK complexes containing the R225Q $\gamma 3$ subunit have a higher basal AMPK activity and lack AMP dependence. Thus, R225Q can be considered a loss-of-function mutation that abolishes allosteric regulation by AMP/ATP, causing increased basal AMPK activity.

The functional significance of the AMPK $\gamma 3$ subunit must be interpreted in light of its very specific tissue distribution, in contrast to the broad tissue distribution of other AMPK subunits (20). Ablation of AMPK $\gamma 3$ leads to impaired AICAR-induced glucose uptake and to reduced glycogen resynthesis after exercise. The R225Q mutation may not directly alter the accumulation of glycogen in skeletal muscle, as glucose uptake and tolerance are unaltered in AMPK $\gamma 3^{\text{R225Q}}$ mice. Rather the R225Q mutation may indirectly alter glycogen storage by altering skeletal muscle oxidation. Evidence for a role of the R225Q mutation in increasing fatty acid oxidation is revealed when AMPK $\gamma 3^{\text{R225Q}}$ mice are challenged with a fat-rich diet. This is paradoxical because expression of the endogenous $\gamma 3$ isoform is restricted to glycolytic muscles. However, a major role of the AMPK $\gamma 3$ isoform may be to ensure that glycogen content in glycolytic skeletal muscle is restored, maintaining a high glycolytic potential through shifting the metabolic fate of fuel toward fat oxidation and glycogen storage.

We provide a biological validation of the muscle-specific $\gamma 3$ isoform as a putative drug target for the prevention of triglyceride accumulation and the development of insulin resistance in skeletal muscle. Targeting the AMPK $\gamma 3$ isoform offers an entry point for tissue-specific regulation of glucose and lipid metabolism in skeletal muscle. A compound mimicking the effect of the R225Q mutation may be efficacious in the treatment of type II diabetes mellitus.

Acknowledgments—We gratefully acknowledge Dr. Fatima Bosch for the pMLC vector used for the transgene constructions, Abhilasha Gupta and Dr. Bryce van Denderen for human AMPK $\alpha 2$ and $\gamma 3$ expression constructs, respectively, Dr. David Carling for discussions, and the Mouse Camp, Karolinska Institutet, and Uppsala University

Transgene Facility for generating transgenic and knock-out mice, respectively.

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The 5'-AMP-activated Protein Kinase γ 3 Isoform Has a Key Role in Carbohydrate and Lipid Metabolism in Glycolytic Skeletal Muscle

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J. Biol. Chem. 2004, 279:38441-38447.

doi: 10.1074/jbc.M405533200 originally published online July 6, 2004

Access the most updated version of this article at doi: [10.1074/jbc.M405533200](https://doi.org/10.1074/jbc.M405533200)

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