Genetic model for the chronic activation of skeletal muscle AMP-activated protein kinase leads to glycogen accumulation

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Barré L, Richardson C, Hirshman MF, Brozinick J, Fiering S, Kemp BE, Goodyear LJ, Witters LA. Genetic model for the chronic activation of skeletal muscle AMP-activated protein kinase leads to glycogen accumulation. Am J Physiol Endocrinol Metab 292: E802–E811, 2007. First published November 14, 2006; doi:10.1152/ajpendo.00369.2006.—The AMP-activated protein kinase (AMPK) is a heterotrimeric protein, consisting of an α-subunit, a β-subunit important both for enzyme activity and targeting, and a γ-subunit, which binds the activating AMP. Activity requires phosphorylation of the α-subunit on Thr172 by one or more AMPKks (24, 26, 27, 33).

To exploit the possibility that a mutation in a γ-subunit might create a model of chronic AMPK activation and, reasoning that the γ1 subunit was the most widely expressed γ-subunit, we previously examined the impact of a mutation in the γ1 subunit (R70Qγ1) on AMPK activity in cell-based systems (18). Three AMPK γ-subunits (γ1, γ2, γ3), are coded by three separate genes, γ1 being the most ubiquitously expressed, whereas γ2 and γ3 have more restricted expression (8, 24, 26, 27). The γ-subunits bind AMP, activating the heterotrimer; however, mutations in the AMP binding pocket of γ1 can create an activated heterotrimer that is relatively independent of allosteric regulation by AMP (1, 18). Naturally occurring mutations in γ2 and γ3 are now well recognized, the former contributing to the arrhythmias and cardiomyopathy of the Wolff-Parkinson-White syndrome (2). The Hampshire pig (Sus scrofa domesticus; RN−) is an interesting model of the impact of a γ3 subunit mutation (R200Q) in skeletal muscle, leading to marked glycogen accumulation (11, 12, 29). This phenotype has been replicated in a murine model with an identically placed mutation in the mouse γ3 subunit (R225Q) (3–5, 38). However, the R225Qγ3 mutation actually decreases skeletal muscle AMPK activity (38).

As studied with reconstituted heterotrimer and in stably expressing cell lines, an R70Qγ1 mutation was found to increase AMPK activity threefold accompanied by a marked increase in AMP-independent activity and heightened Thr172 phosphorylation of the α-subunit (1, 18). Furthermore, AMPK activity in the basal state approximated that of the 5-aminoimidazole-4-carboxamide-1β-D-ribofuranoside (AICAR)-stimulated state in control lines and was accompanied by down-stream AMPK actions, including the alternations in acetyl-CoA carboxylase (ACC) phosphorylation and in the activity/phosphorylation of the p70 S6 kinase expected with endogenous AMPK stimulation (18, 25). An R70Qγ1-containing heterotrimer can also mimic the effects of enhanced AMPK activity on the cystic fibrosis transmembrane conductance regulator (16). Since the R70Qγ1 subunit can associate with either the α1 or α2 isofrom and since γ1-containing heterotrimers are abundantly expressed in all tissues studied to date, this mutation was chosen to create a genetic model of chronic AMPK activation in skeletal muscle that might be extrapolated to other tissue and cell types.

MATERIALS AND METHODS

Animals. Full-length R70Qγ1 cDNA, tagged at the 5′ end with a hemagglutinin (HA) epitope, was cloned into the multicloning site of the vector pACTSV40, which was generously donated by Dr.

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Rabinder Prinjha (6, 13). The pACTSV40 vector contains the skeletal muscle α-actin promoter, which allows expression of the R70Qy1 transgene predominantly in fully differentiated skeletal muscle with some expression in cardiac myocytes. The promoter, HA-γ1R70Q cDNA and SV40 sequence were excised from the vector with the restriction enzyme BamHI and the 3.6-kb linear fragment gel purified and dialyzed in Tris-ErTA buffer with a Slide-a-lyzer 10K Cassette (Pierce, Rockford IL). Microinjection of the purified linear construct into the pronuclei of fertilized eggs derived from inbred FVB/J mice was performed in the Dartmouth Transgenic facility.

Founders were identified by polymerase chain reaction (PCR). Mouse tail DNA for transgene screening was prepared with the High Pure PCR Template Preparation Kit (Roche Diagnostics). Primers for detection of a 650-bp fragment of the transgene were 5’-GCC GTT GGG AGG GGA CAG-3’ and 5’-GGA GAA ATG CAG ACA AGC GG-3’. The upstream primer sequence was specific for the 3’ end of the α-actin promoter and the downstream primer specific for the 5’ end of γ1L. The copy number of total γ1L gene (wild type + transgene) in tail DNA was estimated by real-time PCR using another primer set within the coding region of γ1L cDNA. All the founders were bred to wild-type FVB mice and offspring analyzed by PCR and immunoblotting to confirm germ-line transmission. All of the mice used in the present experiments were heterozygous for the expressed transgene, were from generations f2 to f4, and were housed in a pathogen-free facility with a 12:12-h light-dark cycle and standard chow ad libitum. For cross-breeding experiments, R70Qy1 homozygous mice were mated with homozygous mice selectively expressing a dominant negative AMPK α-subunit (a kind gift of Morris Birnbaum, University of Pennsylvania) (31, 39). The data herein obtained with these cross-bred mice were similar after one and two generations of back-crossing.

All of the animal procedures used in the experiments were approved by the Institutional Animal Care and Use Committee of Dartmouth College and Dartmouth Medical School and of the Joslin Diabetes Center.

Exercise protocols. Overnight-fasted mice were studied between 9 and 12 wk of age in the resting state and after two different exercise protocols. In the swimming exercise protocol, wild-type or transgenic and 12 wk of age in the resting state and after two different exercise protocols. In the swimming exercise protocol, wild-type or transgenic mice swam together in a water bath measuring 33 cm × 31 cm × 31 cm. Water temperature was maintained at 34–35°C. Mice swam for four 30-min intervals separated by 5-min rest periods. After the last swim interval, all mice were killed by CO2 narcosis, and tissues were immediately removed for analysis. For the treadmill running exercise protocol, mice were first adapted to treadmill (Quinton model 42) running through exposure to 5–10 min of low-speed treadmill exercise per day for 3 days. Five hours before the experiment, food was removed, and animals were subjected to a graded treadmill running protocol that resulted in their eventual exhaustion and removal from the treadmill. Mice were placed on the treadmill, and running was initiated at 5.4 m/min, 0° incline. Treadmill speed and incline were increased 5.4 m/min and 2° incline every 3 min thereafter until a 21.6 m/min treadmill speed and a 14° incline was achieved after 21 min. This speed and incline were maintained for an additional 30 min, at which time the incline was increased 2° every 3 min until a 26° incline was achieved. This speed and incline were maintained until the last mouse stopped running from exhaustion.

Mice were considered exhausted when, after considerable prodding with compressed air, they would not leave the electrified grid to return to the treadmill and would not right themselves when placed on their backs. Wild-type mice killed following this type of testing typically, in our hands, display a liver and muscle glycogen content ~30% of sedentary mice. Maximal exercise tolerance was determined by the cumulative amount of work (joules; J) that the mice performed, calculated as body weight (kg) times the sum of distance and vertical distance covered (m) × 9.81.

Tissue extraction, immunoblotting, immunoprecipitation, and AMPK activity. Tissues were rapidly removed from euthanized animals, snap-frozen in liquid nitrogen, and stored at −80°C until used (within 1 wk). Animals were euthanized by CO2 narcosis per the policies of the IACUC at Dartmouth. Extracts were prepared by homogenization in an ice-cold 1% Triton X-100-containing buffer (20), using an Ultra-Turrax homogenizer at top speed for 30 s followed by centrifugation at 14,000 g for 15 min at 4°C. Extract protein content was measured by BCA assay (Pierce). Immunoblotting of extracts (normalized for total protein) after SDS gel electrophoresis was performed as previously described (18). Primary antibodies employed in this study included anti-peptide antibodies that we have previously characterized [anti-AMPK-pan-α (reactive with both α-isoforms), antibodies specific for α1, α2, β1, β2, γ1, and γ3 AMPK subunits, anti-AMPKα p-Thr172, anti-ACC p-Ser79, and anti-HA]. Total ACC content was measured by blotting with streptavidin-horseradish peroxidase. GSY isoform expression and phosphorylation were assessed on immunoblotting with antibodies raised against the muscle glycogen synthase (GSY1; a kind gift of John Lawrence, University of Virginia) and against peptides of the liver isoform (GSY2) and its phosphorylated form (GSY2p). These antibodies were raised in rabbits against the peptide sequences VPPSPGSQASSPSQDDVEDE and VPPSPSVPQASAQPSQSPQDVEDE, respectively, and were immunopurified using the specific antigen. The anti-muscle GSY antibody does not distinguish between GSY1 and GSY2.

Glycogen, metabolite, and enzyme assays. Glycogen content was measured in ~10 mg of skeletal muscle (quadriceps, gastrocnemius, tibialis anterior, and soleus), heart muscle, and liver tissue dissected from overnight-fasted euthanized mice and flash-frozen in liquid nitrogen. The tissue was weighed and then boiled in 2 M HCl to hydrolyze glycogen into glucose. After 3 h of boiling, the hydrolysate was quenched by adding a stoichiometric equivalent of KOH to produce a final tissue concentration of 10.0 mg/ml. The supernatant was separated from the cell debris and then assayed for glucose content with the Infinity Hexokinase assay reagent (Thermotrace). Glucose 6-phosphate (G-6-P), ATP, and phosphocreatine were measured in neutralized perchloric acid extracts, prepared, and assayed as in Ref. 28.

GSY was assayed in the presence and absence of G-6-P (6.7 mM) in extracts prepared by homogenization of muscle in Tris-Cl (50 mM, pH 7.8), EDTA (5 mM), and NaF (100 mM). Enzyme activity is expressed as millimolars per milligram of lystate protein, where 1 μmol = 1 nmol UDP-glucose incorporated into glycogen per minute (32). Glycogen phosphorylase was assayed in lysates prepared in MES (50 mM, pH 6.1), EDTA (5 mM), and NaF (100 mM) in the presence and absence of AMP (3 mM); enzyme activity is expressed as millimolars per milligram of lystate protein where 1 μmol = 1 nmol G-1-P incorporated into glycogen per minute (15).

Immunoprecipitation of tissue extracts and assay of immunoprecipitates for AMPK activity against SAMS peptide in the presence and absence of AMP (200 μM) after absorption to protein A/G beads was performed as previously published (18).

Transmission electron microscopy. Muscle tissue (~1.0 mm3) was fixed for 3 h in 3% glutaraldehyde-2% parafomaldehyde in 0.1 M Na-cacodylate, pH 7.4, at room temperature. They were postfixed in 1% OsO4 in 0.1 M Na-cacodylate, pH 7.4, for 1 h at room temperature and then rinsed in 0.1 M Na-cacodylate at room temperature for 1 h. After a rinse in water, the samples were dehydrated with ethanol, rinsed with propylene oxide, immersed in LXR12:PO (3:2 by volume), dried overnight, and polymerized at 60°C overnight. Microscopy was conducted at the Dartmouth College Electron Microscope Facility.

Analysis of mRNA by DNA microarray and by quantitative real-time PCR. RNA was extracted from snap-frozen skeletal muscle by homogenization in TRIZol (Invitrogen) according to the manufacturer’s protocol. RNA was purified with the RNasy Mini Kit (Qiagen).
clean-up protocol including an on-column DNase digestion step using the RNase-free DNase set per the Qiagen protocols. RNA integrity was verified by denaturing agarose gel electrophoresis and ethidium bromide staining. RNA concentration was determined by measuring the absorbance at 260 nm, and RNA purity was estimated from the ratio of absorbance at 260 and 280 nm.

For the DNA microarray, RNA was prepared from both wild-type and transgenic mouse quadriceps muscles (n = 3 for each). The total mRNAs were then processed following the standard one-cycle eukaryotic target preparation protocol from Affymetrix at the Dartmouth core facility. Briefly, biotinylated cRNA targets were generated from the mRNA and hybridized to GeneChip Mouse Genome 430A arrays (Affymetrix), stained with streptavidin-phycocerythin in the GeneChip Fluidics station, and scanned using an Affymetrix GeneChip Scanner. The array image data were acquired and the fluorescent signal intensities quantified using Affymetrix MAS 5.0 software. The generated cell intensity files and analysis output files were then imported into the GeneTraffic-Uno software (Iobion) for further analysis. A significant difference between wild-type and transgenic samples was arbitrarily designated as a twofold change.

To verify the results from microarray analysis, quantitative real-time PCR was performed. RNA was obtained from wild-type and transgenic mouse muscles as described above, and complementary DNA (cDNA) was generated by reverse transcription of total RNA by use of random hexamer primers and MultiScribe reverse transcriptase, following the recommended protocol from Applied Biosystems. PrimerExpress software (v. 2, Applied Biosystems) was used to design the real-time PCR primers and dual-labeled fluorescent probes for murine mRNAs corresponding to GSY1 (forward primer 5'-GGA TGT TGC AGC CTC AGC TT-3', reverse primer 5'-GAT GTT GCA GGT GTC CCA AAG-3', and probe 5'-CCC TGG AGC GAT GGA AGG GTG AAA-3') and GSY2 (forward primer 5'-TCC CTG TGG AAG ACT TAC TGC TT-3', reverse primer 5'-TTG GCC TTG GTC TGG ATC A-3', and probe 5'-TTC TTG GGA GTG GAC CAA CAA AGT TGG G-3'). Preliminary experiments revealed that no PCR product was observed. Decreased expression of the endogenous AMPK was roughly consistent with the increase in gene copy number altering between 95°C for 15 s and a 60°C elongation step for 1 min. Data were analyzed using Sequence Detection System software v. 1.9 (Applied Biosystems). Threshold cycles (C_T) were determined for all reactions; data are presented as the ratio of observed C_T for the mRNA of interest to that of the C_T for GAPDH to correct for small differences due to varying sample DNA concentration.

Statistical analyses. Where applicable, results were compared by Student’s t-test, ANOVA, or the Wilk’s λ approximation of F-test.

RESULTS

Expression of the R70Qγ1 transgene and effects on endogenous AMPK. Three founder lines incorporating the HA-R70Qγ1 transgene were identified by PCR screening of genomic DNA; real-time PCR revealed an approximately threefold increase in total γ1 gene copy number compared with wild-type mice (data not shown). This report focuses entirely on mice heterozygous for the transgene, as analyzed at 9–12 wk of age; all data were confirmed in two separate founder lines. No impact of transgene expression was seen on litter size; expected male/female ratio; body weight (±20 wk of age); skeletal muscle, heart, and adipose tissue weight; or fasting blood glucose or insulin levels (data not shown).

Immunoblotting of tissue extracts with anti-HA antibody confirmed muscle-specific expression of the HA-R70Qγ1 protein of 40 kDa in gastrocnemius, tibialis anterior, quadriceps, and extensor digitorum longus muscles, although there was little detectable expression in soleus (Fig. 1A). There was lower-level expression in heart but none in spleen or 12 other tissues, including adipose tissue, liver, and brain (not shown). Blotting with an anti-γ1 antibody revealed an approximately twofold increase in total γ1 protein in skeletal muscle (Fig. 1B) roughly consistent with the increase in gene copy number observed. Decreased expression of the endogenous AMPKγ3

![Image](http://ajpendo.physiology.org/DownloadedFrom/10.220.32.246)
subunit was observed in skeletal muscle (Fig. 1B). No changes in total AMPK α-subunit (Fig. 1B) or in α1, α2, or β2 protein content were seen (data not shown).

The expressed HA-R70Qγ1 protein was incorporated into AMPK heterotrimers with endogenous α- and β-subunits. Immunoprecipitation of lysates from quadriceps, gastrocnemius, and heart with anti-HA antibody led to the coprecipitation of AMPKα (Fig. 2A), whereas immunoprecipitation with a pan-α antibody led to coprecipitation of HA-R70Qγ1 (Fig. 2B). The α-subunit was appropriately phosphorylated on Thr172, a prerequisite for enzyme activity (Fig. 2C). By specific immunoblotting, HA-R70Qγ1 was found to associate with both α1- and α2-containing heterotrimers; these heterotrimers contained only the β2 and not the β1 subunit (data not shown). AMPK activity was measured after immunoprecipitation with anti-pan-α- or anti-α-specific antibodies. Total AMPK activity (measured after pan-α immunoprecipitation of heterotrimers containing both α-isoforms) was increased ~2.1-fold in immunoprecipitates from rested transgenic skeletal muscle compared with wild-type muscle (Fig. 3A). Both α1- and α2-containing heterotrimers contributed to this increase in AMPK activity (Fig. 3, C and D), which was also accompanied by a corresponding increase in phosphorylation of the α-subunit on Thr172 (Fig. 3B). When immunocomplexes were assayed in the presence and absence of AMP in the kinase reaction, there was a marked increase in the fraction of total AMPK activity that was AMP independent in anti-pan-α immunoprecipitates from transgenic muscle extracts (gastrocnemius: Tgn 48 ± 2%, WT 29 ± 2%; quadriceps: Tgn 58 ± 2%, WT 31 ± 6%, n = 3 each; P < 0.001). The magnitude of these increases in AMPK activity and the fraction of AMP-independent activity approximates that previously seen on expression of the HA-R70Qγ1 in stable cell lines and with reconstituted heterotrimer (1, 18). No alterations in total AMPK activity were noted either in tissues that did not express the transgene (soleus, liver) or in cardiac muscle, where there were lower levels of expression (data not shown).

Alterations in glycogen content and requirement for an active AMPKα. The glycogen content of quadriceps, gastrocnemius, and tibialis anterior was increased two- to threefold in HA-R70Qγ1-expressing transgenic mice (Fig. 4A), whereas the glycogen content of tissues from transgenic animals that either did not express the transgene (liver, soleus) or expressed at low levels (cardiac) was unaltered (Fig. 4B). Transmission electron microscopy revealed increased sarcoplasmic glycogen accumulation in quadriceps (Fig. 4, C and D) and in gastrocnemius (data not shown) of transgenic animals.

To ascertain whether the increase in glycogen content in these transgenic mice required the activation of endogenous AMPK activity and was not due to some other action of the HA-R70Qγ1 protein product unrelated to its incorporation into an AMPK heterotrimer, HA-R70Qγ1-expressing mice were cross-bred with mice expressing a dominant negative α-subunit [which results in a marked decrease in the expression of the native α-subunit protein (31, 39)]. Estimation of the glycogen content in the gastrocnemius of the offspring of these matings...
revealed that the increase in glycogen content seen in progeny expressing the R70Q1 subunit alone was substantially normalized in mice coexpressing the dominant negative α-subunit, in which no detectable endogenous α-subunit is present (Fig. 5). Confirming a prior result, offspring expressing only the dominant negative α-subunit had a small decrease in glycogen content compared with wild-type animals (31, 39).

Effect of activated AMPK on genes involved in glycogen metabolism. Because AMPK has been implicated as a regulator of multiple aspects of cellular glucose metabolism, we have examined several of the potential loci by which the activated R70y1-AMPK heterotrimers in the transgenic animals might be contributing to the observed increase in skeletal muscle glycogen content. To screen for possible alterations in gene expression, DNA microanalysis of mRNA extracted from the quadriceps muscle of wild-type and transgenic mice was performed. Limited analyses to date have revealed no apparent changes in the levels of mRNAs encoding GLUT4, hexokinases I and II, muscle glycogen phosphorylase, or the branching enzyme in the transgenic animals. There was also no apparent change in the mRNA encoding the muscle glycogen synthase isozyme (GSY1); but, unexpectedly, we noted a 7.3-fold increase in mRNA encoding the liver glycogen synthase isozyme (GSY2; data not shown). This finding was confirmed, with real-time PCR, with an increase in GSY2 mRNA (quantitated as a decrease in the observed C Trotsky1) but not in GSY1 mRNA. [GSY1: WT CT 17.52 ± 0.89 (mean ± SD); Tgn CT 17.00 ± 1.44 (P = 0.6); GSY2: WT CT 23.56 ± 0.81; Tgn CT 20.02 ± 0.80 (P = 0.003)].

Extracts of skeletal muscle from wild-type and transgenic mice were next examined by immunoblotting, employing antibodies directed against these two GSY isozymes and against the phosphorylated form of GSY2. GSY2 is undetectable in wild-type muscle and is markedly induced (~80 kDa) in the transgenic muscle. Multiple closely migrating 80-kDa GSY2 bands, presumably reflecting differential phosphorylation, are observed in liver, but their expression is the same in wild-type and transgenic liver (Fig. 6, top). Blotting with an antibody specific for the phosphorylated form of GSY2 additionally indicates a marked increase in detectable GSY2 in R70Qy1 transgenic muscle, reflecting either increased GSY2 content or phosphorylation, or both. There are no apparent differences in p-GSY2 in the liver (Fig. 6, middle). There is no clear change in total GSY (GSY1/2) protein in the skeletal muscle of transgenic mice, as detected with the antibody raised against the intact skeletal muscle enzyme (Fig. 6, bottom). However, the GSY1/2 protein is shifted to slower migration on SDS-PAGE, likely consistent with increased GSY phosphorylation, as predicted from in vitro observations of phosphorylation of GSY by AMPK (17, 23).

We next measured the activities of GSY (in the presence and absence of the allosteric regulator G-6-P) and glycogen phos-

Fig. 3. AMPK activity and Thr172 phosphorylation: A: immunocomplexes of AMPK, precipitated with anti-AMPK-pan-α, were assayed for AMPK activity in the presence of a saturating concentration of AMP (20). Extracts for these assays were prepared from the gastrocnemius of WT (open bars) or Tgn (filled bars) mice in the resting state and after 2 h of swimming exercise. AMPK activity is expressed as pmol 32P·min⁻¹·mg immunoprecipitated (IP’d) protein⁻¹ transferred to SAMS peptide. Each bar represents the mean (SD) from n = 4 animals; *difference in the resting activity between WT and Tgn and in effect of exercise on the WT activity is significant by ANOVA (P < 0.002). B: detergent extracts from n = 4 mice (WT, open bars; Tgn, filled bars) were probed with anti-α-T172p antibody. *P < 0.01 by Student’s t-test. C and D: immunocomplexes of AMPK from WT (open bars) or Tgn (filled bars) gastrocnemius extracts were precipitated with an anti-AMPKα1- (C) or anti-AMPKα2-specific antibodies (D) and were assayed for AMPK activity as above. Data are expressed as pmol 32P·min⁻¹·mg IP’d protein⁻¹ transferred to SAMS peptide. *P < 0.01 by Student’s t-test for n = 4 of each.
phorylase (in the presence and absence of the allosteric regulator AMP) in extracts from wild-type and skeletal muscle. There was a small, but significant, increase in GSY activity, measured in the absence or presence of G-6-P, in R70Q/H92531-transgenic muscle (Fig. 7A). Glycogen phosphor-
ylase activity in the absence or presence of AMP was unaltered (Fig. 7B).

As measured in perchloric acid extracts, the ATP and phosphocreatine content of R70Q/H92531-expressing muscle was similar to wild-type skeletal muscle. However, a 50% decrease in G-6-P content was observed (Table 1). Glucose uptake (employing 2-deoxy-[3H]glucose), measured in isolated EDL muscle under basal conditions (38), was reduced (by ~40%) in transgenic muscle (Table 1).

Comparison of sedentary and exercised wild-type and trans-
genmic mice. As AMPK is activated in skeletal muscle during both acute and chronic exercise associated with the subsequent phosphorylation of its downstream target, ACCβ, it was of interest to compare these parameters in wild-type and transgenic mice in the rested state and after 2 h of exercise (swimming; see SUPPLEMENTAL METHODS, online version). As expected, AMPK activity (Fig. 3A), AMPKα p-Thr172, and ACCβ phosphorylation (Fig. 8, A and B) were increased in response to exercise in wild-type mice. Strikingly, the level of AMPK activity (Fig. 3A) and AMPKα Thr172 and ACCβ phosphorylations (Fig. 8, A and B) in the R70Qγ1-expressing transgenic mice in the resting state was equivalent to or exceeded that observed in wild-type mice after 2 h of exercise. Exercise itself caused little further change in AMPKα Thr172 and ACCβ phosphorylations in the transgenic mice (Fig. 8, A and B).

Consistent with the increase in glycogen content, transgenic mice had an increased exercise tolerance. Mice were adapted to treadmill exercise and then subjected to a graded treadmill running protocol that resulted in their eventual exhaustion and removal from the treadmill. Maximal exercise tolerance, determined by the cumulative amount of work (J) that the mice...
vivo provides a strategy for activating AMPK in other tissues, which may prove useful in delineating other roles for the kinase. To our knowledge, this represents the first in vivo model of tissue-specific AMPK activation employing stably expressing transgenic mice. Others have successfully employed transgene expression techniques using adenoviral-mediated gene transfer, employing constitutively active truncated/mutated AMPKα or AMPKγ subunits (14, 30, 35); however, the transient nature of the expression necessarily limits the overall utility of these models.

We elected not to create a wild-type γ1 subunit-expressing murine line, because our previous observations in cultured cell lines indicated that only the R70Q mutant and not the wild-type γ1, when overexpressed, activated endogenous AMPK (18). Furthermore, we have documented that the heterotrimers containing this mutant form of the γ1 subunit have an increased AMPK specific activity in vitro (18). Similarly, mutations of other key residues in the γ-subunit AMP binding pocket also have increased basal activity (1). This genetic AMPK activation is now also observed in murine skeletal muscle, which was the goal of this study, although we cannot rigorously exclude in this model that there is a contribution of γ1 overexpression. The mechanisms by which γ3 expression is reduced are not known but could involve increased turnover of the non-complexed γ3 in the presence of an excess of the HA-R70Qγ1 mutant. On the basis of a previous report, this reduction is unlikely to contribute to glycogen accumulation (5). The increase in glycogen content does require an increase in AMPK activity (i.e., an activated AMPK α/β/γ heterotrimer).

The changes in glycogen content and skeletal muscle ultrastructure seen in these transgenic mice mirror those observed in both the Hampshire pig, with an R200Q equivalent mutation in the γ3 subunit of AMPK, and in the transgenic mice overexpressing an equivalent R225Qγ3 mutation in skeletal muscle (3–5, 38). However, the porcine R200Qγ3 mutation has been shown to inactivate AMPK, whereas conflicting information has been presented for the murine R225Qγ3 mutation. Based on transfection of COS cells, the R225Qγ3 mutation is reported to activate AMPK (5). However, when the same subunit mutation is expressed transgenically in skeletal muscle, AMPK activity is diminished (38). Mutations in the γ2 subunit causing the Wolff-Parkinson-White syndrome result in intracellular vacuoles containing glycogen in the heart, although these

### Table 1. Measurements of ATP, PCr, G-6-P, and glucose uptake

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<tr>
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<th>ATP</th>
<th>PCr</th>
<th>G-6-P</th>
<th>Glucose Uptake</th>
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<tr>
<td>Wild type</td>
<td>4.7 (1.2)</td>
<td>52 (19)</td>
<td>0.26 (0.05)</td>
<td>2.41 (0.70)</td>
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<tr>
<td>Tgn</td>
<td>5.6 (0.57)</td>
<td>74 (11)</td>
<td>0.14 (0.03)</td>
<td>1.41 (0.22)</td>
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Values are means (SD); n = 4 each. Shown are measurements of ATP, phosphocreatine (PCr), and glucose 6-phosphate (G-6-P) performed in the same extracts of gastrocnemius muscle from wild-type and hemoglobinin (HA)-R70Qγ1 transgenic (Tgn), where data are expressed as μmol/g muscle. Assessed by the Wilk’s λ approximation of the F-test, only the G-6-P difference is statistically significant (F = 12.80, P < 0.012). Glucose uptake in extensor digitorum longus muscle under basal conditions, performed as in Ref. 38 with 2-deoxy-[3H]glucose, is indicated as means (SD) of n = 8 (wild-type) and n = 6 (Tgn) observations, where the data are expressed as μmol uptake·g muscle⁻¹·h⁻¹. By Student’s t-test, the difference between wild-type and Tgn is significant at P < 0.0055.
mutations have also paradoxically been reported to inactivate AMPK (2, 9). In our hands, the R70Q and other mutations in the AMP binding pocket of the CBS domains of the γ1 subunit activate AMPK and result in an enzyme that is relatively independent of the allosteric effects of AMP, as is the case with the R225Q mutation (1, 18). With respect to the present study, this AMP independence would appear to account for a basal activity and phosphorylation state of AMPK and ACC that mimic that of the exercised muscle, where activation of AMPK is due to changes in the ratio [AMP]/[ATP]. The R70Q mutation, then, not only mimics the allosteric actions of AMP but, like AMP, likely increases its ability to be phosphorylated by upstream kinase(s), such as LKB1 and/or the CaMKKs (20–22, 33, 36, 37).

The mechanisms underlying the increase in muscle glycogen content in the R70Qγ1 transgenic cannot be fully clarified from the present study. The reduction in G-6-P content is suggestive of either decreased glucose transport/phosphorylation and/or of increased G-6-P utilization, because of either increased glycolysis or increased glycogen synthesis. The observed reduction in glucose transport may be reflective of the increase in muscle glycogen content under these steady-state conditions, so we cannot rigorously exclude an antecedent increase in glucose uptake contributing to the change in glycogen (10). Studies of glycogen repletion during fasting/refeeding or after sustained exercise using this model will be of interest.

The significance of the unexpected increase in the content of the liver isozyme of GSY (GSY2) in the R70Qγ1-transgenic muscle is unclear. This increase seems in part accounted for by an increase in GSY2 gene expression, although we cannot exclude contributions from alterations in mRNA turnover or in protein turnover (the latter perhaps due to a stabilization of the GSY2 protein on binding to the glycogen pellet). Consistent with other observations of the direct effects of AMPK on skeletal muscle GSY (GSY1), our data indicate an increase in muscle GSY (GSY1/2) phosphorylation state, which would predict a decrease in its activity (17, 23, 34). Similarly, there appears to be an increase in measurable p-GSY2, although this apparent increase is, at least in part, due to increased expression of GSY2 protein. We could not estimate any changes in the ratio of p-GSY2 to total GSY2 in our study, since only the p-GSY2-specific antibody has sufficient sensitivity to detect GSY2 in wild-type mice. However, despite an apparent prediction of decreased overall GSY activity in response to AMPK activation and GSY phosphorylation (17, 23, 34), we have noted a small, but significant, increase. We cannot be certain quantitatively what the relative mix of GSY1 and GSY2 proteins/activities is in these extracts, as we were unsuccessful in using the antibodies for isoform-specific immunoprecipitation assays. The kinetic parameters of these two GSY enzymes are not sufficiently different to permit an estimation of this relative mix by varying assay kinetic conditions. In addition, the response of the kinetic properties of GSY1 and GSY2 (Vmax, S0.5 UDP-glucose, M0.5 G-6-P) to phosphorylation are sufficiently different to potentially mask important in vivo changes in overall GSY activity in a mixture of the two enzymes (7). Nonetheless, the novel observation of the induction of GSY2 by AMPK activation and its potential contribu-
tion to altered glycogen metabolism in skeletal muscle and other nonhepatic organs is deserving of further study.

The expanded glycogen pool observed in our transgenic mice is likely a major contributor to the observed enhanced exercise capacity. Carbohydrate loading by ingestion of carbohydrate-rich nutrients is a well-recognized maneuver among endurance athletes for extending the period of exercise, although its effects on short-term performance are disputed (19). Of interest, AMPK and ACC activity and phosphorylation in the skeletal muscle of our transgenic mice in the resting state have the characteristics of the muscle of exercised animals. Given the likelihood of multiple effects of an activated AMPK on the molecular characteristics of skeletal muscle, this basal activation might also be contributing in other ways to the enhanced exercise capacity (e.g., increased fatty acid oxidation).

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