Cloning and characterization of mouse 5′-AMP-activated protein kinase γ3 subunit

Haiyan Yu, Nobuharu Fujii, Michael F. Hirshman, Jason M. Pomerleau, and Laurie J. Goodyear. Cloning and characterization of mouse 5′-AMP-activated protein kinase γ3 subunit. Am J Physiol Cell Physiol 286: C283–C292, 2004. First published September 24, 2003; 10.1152/ajpcell.00319.2003.—Naturally occurring mutations in the regulatory γ-subunit of 5′-AMP-activated protein kinase (AMPK) can result in pronounced pathological changes that may stem from increases in muscle glycogen levels, making it critical to understand the role(s) of the γ-subunit in AMPK function. In this study we cloned the mouse AMPKγ3 subunit and revealed that there are two transcription start sites, which result in a long form, γ3L (AF525500) and a short form, γ3S (AF525501). AMPKγ3L is the predominant form in muscle and is specifically expressed in mouse skeletal muscle at the protein level. In skeletal muscle, AMPKγ3 shows higher levels of expression in fast-twitch white glycolytic muscle (type Iib) compared with fast-twitch red oxidative glycolytic muscle (type Iia), whereas γ3 is undetectable in soleus muscle, a slow-twitch oxidative muscle with predominantly type I fibers. AMPKγ3 can coimmunoprecipitate with both α and β AMPK subunits. Overexpression of γ3S and γ3L in mouse tibialis anterior muscle in vivo has no effect on α1 and α2 subunit expression and does not alter AMPKα2 catalytic activity. However, γ3S and γ3L overexpression significantly increases AMPKα1 phosphorylation and activity by ~50%. The increase in AMPKα1 activity is not associated with alterations in glycogen accumulation or glycogen synthase expression. In conclusion, the γ3 subunit of AMPK is highly expressed in fast-twitch glycolytic skeletal muscle, and wild-type γ3 functions in the regulation of α1 catalytic activity, but it is not associated with changes in muscle glycogen concentrations.

adenosine 5′-monophosphate-activated protein kinase; AMPKγ3 short form; AMPKγ3 long form; cystathionine β-synthase domain

THE 5′-AMP-ACTIVATED PROTEIN KINASE (AMPK) functions as a critical sensor of cellular energy charge in a wide range of tissues and cells. AMPK is activated in response to stimuli that decrease ATP and phosphocreatine concentrations (11, 23, 38) by both allosteric and phosphorylation-dependent mechanisms (13, 15, 24, 41). In mammals as well as yeast, AMPK has been proposed to regulate a host of metabolic and transcriptional events, such as glucose transport (21, 26, 30, 46), fatty acid oxidation (16, 37, 42, 48), leptin regulation of lipid metabolism (32), and gene transcription (29, 51). AMPK is a heterotrimer, consisting of one catalytic subunit (α) and two noncatalytic and presumable regulatory subunits (β, γ) (33, 40). Each of the subunits has multiple isoforms, and various configurations of the heterotrimer have been reported, including α1β1γ1, α1β2γ1, α2β1γ1, and α2β2γ1 (10, 44).

Although the two α catalytic subunit isoforms of AMPK have been the major focus of AMPK research over the last 10 years, there is now increasing interest in understanding the roles of the β- and γ-subunits in AMPK function. Three γ-isofoms have been identified, γ1, γ2, and γ3. Northern blot analysis of human tissues reveals that γ1 is widely distributed, whereas AMPKγ2 is also widely distributed but has very high expression levels in heart (11, 31). Interestingly, γ3 is almost exclusively expressed in the skeletal muscle of humans (11, 31). The divergent expression patterns of the γ isoforms among tissues suggest that the different isoforms have tissue-specific functions (11, 44). In comparing the human γ1, γ2, and γ3 amino acid sequences, the NH2 termini have significant variations in length and identity, suggesting that this region may play an important role in conferring isoform specificity either by targeting different downstream molecules or by responding to different upstream stimuli. However, specific functions of the NH2-terminal region have not been identified for all three γ-subunits, and there are no known protein motifs in this region. In contrast, the COOH-terminal region contains four consecutive cystathionine β-synthase (CBS) domains that are highly conserved in all γ isoforms (11, 41). These CBS domains occupy approximately half of the γ-subunit, suggesting that these domains are also critical for γ-subunit function (7). Indeed, mutations of CBS domains in a number of proteins, including the CBS domains of the γ-subunit of AMPK, are associated with various disease states (4, 8, 20, 28, 31). In skeletal muscle and heart, mutations of the CBS domains in γ2 and γ3 have been shown to be associated with alterations in glycogen metabolism (3, 4, 12, 31).

In recent years, skeletal muscle has become a major focus of the AMPK field because AMPK may be involved in the regulation of glucose uptake, glycogen metabolism, fatty acid oxidation, and gene transcription in this tissue (5, 21, 23, 25, 34, 36, 43, 47, 49). Although mutations in γ-subunit CBS domains, including γ3, lead to pathological conditions, very little is known about the mechanisms by which the γ-isofoms may regulate AMPK activity, cellular metabolism, and tissue phenotype. There have been discrepancies in reports of tissue distributions of γ3 mRNA and protein (11, 31), as well as conflicting reports of the translation initiation sites of the human AMPKγ3 gene (11, 31). In this study we have cloned the full-length mouse AMPKγ3 gene and, by determining its translation initiation sites, have revealed that there are two splice variants of γ3. We have also characterized the AMPKγ3 isoforms and investigated the effects of overexpressing the

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wild-type AMPKγ3 subunit in vivo on AMPK activity and glycogen metabolism in skeletal muscle.

**METHODS**

**Materials.** [γ-32P]ATP was obtained from Perkin-Elmer. Affinity purified polyclonal antibody against AMPKγ3 was raised against mouse AMPKγ3 peptide NH2-CLVDETQHLLGV-COOH (γ3L; 457–467), which is also conserved in mouse γ3S, human γ3, and pig γ3. Polyclonal antibodies against AMPKα1 and AMPKα2 were raised against the peptide sequence 339–355 of AMPKα1 and 352–366 of AMPKα2 (35); anti-AMPKα1/2 antibody was raised against the peptide sequence 2–16 of AMPKα1/2 (AEGK RhDGRVKIGHY), which is conserved in AMPKα1 and AMPKα2 isoforms. Anti-phospho-AMPKα antibody was from Cell Signaling Technology; anti-glycogen synthesis antibody was provided by Dr. John C. Lawrence, Jr. (University of Virginia, Charlottesville, VA); anti-phospho-glycogen synthase antibody was from Oncogene Research Products; the pCAGGS vector was a gift from Dr. J. Miyazaki (Osaka University, Osaka, Japan); and pMT-AMPKβ1 vector and anti-AMPKγ1 antibody were provided by Dr. Lee A. Witters (Dartmouth Medical School, Hanover, NH).

**Animals.** Female (8 wk) ICR mice (30 g) were purchased from Taconic. Protocols for animal use were reviewed and approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.

**Human muscle.** The experiment using a human muscle lyses study with human subjects was approved by the Ethical Committee at the Karolinska (35).

**Cloning of mouse AMPKγ3 cDNA.** Total RNA was extracted from 100 mg of mouse skeletal muscle using Tri Reagent (Molecular Research Center). Total RNA (5 μg) was subjected to the oligo(dT) primer reverse transcription (RT) reaction (Invitrogen), and the cDNA products were used as a source for polymerase chain reaction (PCR) templates. The primers were designed on the basis of conserved regions of pig and human AMPKγ3: sense, 5′-gctgtagtcacgaccggtg-3′; and antisense, 5′-gcggccgcgccaccatggagcccgagctggagcacac-3′. The PCR product was subcloned into pCR 2.1-TOPO vector (Invitrogen) and sequenced. To obtain the upstream extension of 5′-end and the downstream extension of 3′-end of RT-PCR product, 5′- and 3′-rapid amplification of cDNA ends (RACE) was performed using a Marathon-Ready cDNA kit (Clontech). The antisense primer for 5′-RACE, 5′-agccatggcatcataacaggtgtgttcc-3′, and sense primer for 3′-RACE, 5′-ctggaggagttcttcggtc-3′, were designed by the DNA sequence of RT-PCR product as described above. 5′-RACE was performed between the anchor sense primer AP1 (Clontech) and the 5′-RACE antisense primer. The resulting PCR products were reamplified by using the nested sense primer AP2 (Clontech) and the same reverse primer, and the PCR products were cloned into pCR 2.1-TOPO vector for cloning and sequencing. 3′-RACE was performed between the 3′-RACE sense primer and anchor antisense primer AP1, and the resulting PCR products were reamplified by using the same sense primer and nested antisense primer AP2. The PCR products were also cloned into pCR 2.1-TOPO vector and further sequenced.

**Cloning of mouse genomic AMPKγ3.** An AMPKγ3L cDNA fragment (246–822) was used as a probe to screen the mouse genomic DNA library (IncyteGenomics). The γ3-containing clones were confirmed by Southern Blotting using the same probe. The genomic organization was identified by comparing cDNA and genomic sequences.

**Muscle processing.** Muscles were dissected and snap frozen in liquid nitrogen. The pulverized samples were weighed and Polytron homogenized (Brinkmann Instruments) in ice-cold lysis buffer (1:10, wt/vol) containing 20 mM Tris-HCl (pH 7.4), 1% Triton-X 100, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol (DTT), 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSF, followed by centrifuging at 14,000 g for 20 min at 4°C. Supernatants were removed and used for protein concentration measurements, Western blotting, and AMPK activity assay.

**Cell culture.** L6 myoblast cells (1 × 10^6 cells; gift from Dr. Amira Klip, University of Toronto, Toronto, ON, Canada) were plated on 60-mm dishes and grown in α-MEM with 10% fetal bovine serum. Each plasmid DNA (7 μg) was introduced into cells with Lipofectamine 2000 reagent (Invitrogen). Cells were then cultured for another 48 h and then harvested with ice-cold AMPK lysis buffer.

**Immunoblotting.** Protein (20 μg) from cells and muscle lysates was separated by 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in Tris-buffered saline with 0.05% Tween 20 (TBST) and 5% nonfat milk for 1 h at room temperature. The membranes were incubated overnight at 4°C with the appropriate primary antibodies. Bound primary antibodies were detected with anti-rabbit (Amersham) or mouse immunoglobulin-horseradish peroxidase-linked whole antibody (Transduction Laboratories, Lexington, KY). The membranes were washed with TBST and then incubated with enhanced chemiluminescence reagents (Perkin-Elmer) and exposed to film. Bands were visualized and quantified using Image-Quant software (Molecular Dynamics).

**mRNA levels of AMPKγ3L and AMPKγ3S.** The mRNA levels were determined on the basis of PCR. cDNA was synthesized from mouse quadriceps muscle mRNA by reverse transcription, adaptor-ligated double-strand cDNA was used as a template (Clontech), and the adaptor sequence (5′-ccatctaatagcactatagucggttcgg3′) was attached to the 5′-end of cDNA including both γ3S and γ3L cDNA was used as the sense primer. The sequence 5′-ggccagcttcaacaagctggttcggg3′, conserved in both γ3L and γ3S, was used as the antisense primer. The two resulting products from the unsaturated PCR reaction were separated by 1% agarose gel and confirmed by DNA sequencing.

**Immunoprecipitation.** Muscle lysates (200 μg) were incubated with anti-AMPKγ1/2 or -AMPKβ antibody-bound protein A/G beads (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The immunocomplex was washed with lysis buffer and boiled in Laemmli’s buffer. The samples were separated by 8% SDS-PAGE, transferred to nitrocellulose membranes, and then immunoblotted with appropriate antibodies.

**AMPK activity assay and glycogen content.** AMPK activity assay was performed as described by Musi et al. (35). Briefly, muscle lysates were immunoprecipitated with specific antibodies to the α1 and α2 catalytic subunits of AMPK and protein A/G beads. Immunoprecipitates were assayed for AMPK activity in assay buffer containing 1.0 mM ATP, 0.2 mM AMP, 0.2 mM NaF, 50 mM NaCl, 250 mM sucrose, 50 mM NaF, 5 mM sodium pyrophosphate, 2 mM dithiothreitol, 4 mg/l leupeptin, 50 mg/l trypsin inhibitor, 0.1 mM benzamidine, and 0.5 mM PMSF, followed by centrifuging at 14,000 g for 20 min at 4°C. Supernatants were removed and used for protein concentration measurements, Western blotting, and AMPK activity assay.

**Generation of recombinant AMPKγ3.** The coding region of AMPKγ3S or AMPKγ3L was obtained by PCR with the sense primer spanning the start codon containing a Kozak sequence, with or without a Flag-tag sequence and antisense primer spanning the stop codon. The purified PCR product was subcloned into pCR 2.1-TOPO vector and sequenced. The Ecol fragment containing the AMPKγ3 in the pCR 2.1-TOPO vector was excised and subcloned into the pCAGGS vector. Sense primers were as follows: γ3S, 5′-ggccacggtcagcactatagucagctggttcggg3′; γ3L, 5′-ggccacggtcagcactatagucagctggttcggg3′; Flag-γ3S, 5′-ggccacggtcagcactatagucagctggttcggg3′; and Flag-γ3L, 5′-ggccacggtcagcactatagucagctggttcggg3′. The antisense primer for γ3S, Flag-γ3S, γ3L, and Flag-γ3L was 5′-ggccacggtcagcactatagucagctggttcggg3′.

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Expression of AMPKγ3S and AMPKγ3L in tibialis anterior muscle. DNA injection and in vivo electroporation were done by a modification (18) of the method of Aihara and Miyazaki (2). Mice were anesthetized with pentobarbital sodium (90 mg/kg body wt ip), and 100 μg of pCAGGS-AMPKγ3S, pCAGGS-AMPKγ3L, or an equal amount of empty pCAGGS vector in 25 μl of saline was injected into the tibialis anterior (TA) muscle, using an insulin syringe with a 29-gauge needle. For the control, an equal amount of pCAGGS alone was injected into the opposite leg. With the use of an electric pulse generator, square-wave electrical pulses (200 V/cm) were applied eight times at a rate of 1 pulse/s with each pulse being 20 ms in duration. The electrodes used were a pair of stainless steel needles inserted and fixed 5 mm apart into the TA muscle. Nine days after gene delivery, the muscles were removed and prepared for AMPK activity, Western blotting, and the measurements of glycogen content.

Muscle contraction. pCAGGS-AMPKγ3S, pCAGGS-AMPKγ3L, or an equal amount of empty pCAGGS vector was injected into the TA muscle, followed by electroporation. Nine days later, mice were anesthetized with pentobarbital sodium (90 mg/kg body wt ip), the sciatic nerves of both legs were surgically exposed, and subminiature electrodes were attached to the nerves (22, 39). Hindlimb muscles of one leg were electrically stimulated to contract for 20 min (train rate 1/s, train duration 500 ms, rate 100 pulses/s, duration 0.1 ms). The red fast-twitch oxidative-glycolytic muscle, which is functionally expressed in skeletal muscle (11, 31). Interestingly, white fast-twitch glycolytic skeletal muscle that contains predominantly AMPKγ3 is highly expressed in gastrocnemius muscle, with no detectable signal in heart, brain, lung, liver, kidney, pancreas, spleen, white fat, or brown fat (Fig. 2A). These results are consistent with Northern hybridization data from human tissues, which showed that AMPKγ3 is specifically expressed in skeletal muscle (11, 31). Interestingly, white fast-twitch glycolytic skeletal muscle that contains mostly type IIb fibers (white quadriceps and white gastrocnemius) showed higher levels of AMPKγ3 expression compared with the red fast-twitch oxidative-glycolytic muscle, which contains primarily type IIa fibers (red quadriceps and red gastrocnemius). AMPKγ3 was not detectable in soleus, a muscle that is composed almost exclusively of red slow-twitch oxidative type I fibers. This expression pattern suggests that AMPKγ3 plays a unique role in skeletal muscle, especially in the more fast-twitch glycolytic fiber types.

Immunoblotting of mouse skeletal muscle (quadriceps) and human skeletal muscle with γ3 antibody reveals a single band (Fig. 2B). To determine whether γ3S or γ3L is expressed in mouse and human skeletal muscle, we overexpressed recombinant mouse γ3S and γ3L in L6 myoblast cells that do not express endogenous AMPKγ3. Cell lysates were compared with endogenous γ3 from mouse and human muscle lysates. As shown in Fig. 2B, mouse endogenous AMPKγ3 had the same molecular size as γ3L, suggesting that AMPKγ3L is the predominant form in mouse muscle. On the other hand, human AMPKγ3 had the same molecular size as γ3S, although this finding will need to be confirmed in future studies using overexpressed human γ3. The double band on SDS-PAGE of

RESULTS

Cloning mouse AMPKγ3 gene. The full-length sequence of the mouse AMPKγ3 gene was obtained by a combination of RT-PCR and 5′- and 3′-RACE. Sequence analysis of the 1,197-bp fragment from RT-PCR revealed a sequence similar to human and pig AMPKγ3 that was used to design primers for both 5′- and 3′-RACE. The 3′-RACE products were 1,500 bp; sequencing and contiguous analysis revealed that they overlapped with the RT-PCR core 1,197-bp fragment. This demonstrated that the fragment from 3′-RACE is an extension of the core fragment of the mouse AMPKγ3 gene. The 5′-RACE product contained 600- and 630-bp bands. Sequencing and contiguous analysis revealed that the two fragments shared the same sequence at their 3′-region and that this region also overlapped with the core 1,197-bp fragment of mouse AMPKγ3 gene, with the remaining 5′-regions being different (Fig. 1B). The RT-PCR and 3′- and 5′-RACE results suggest that there are two variants of the mouse AMPKγ3 gene that have distinct 5′-regions but are identical throughout the remainder of the gene. Sequence analysis showed that variant 1 contains a 1,392-bp open reading frame. The initiation methionine in variant 1 was designed by an upstream in-frame stop codon and the Kozac sequence surrounding it, and no obvious poly(A) tail or polyadenylation site consensus sequence was found in the 3′-noncoding region. The sequence analysis of variant 2 showed that it contains a 1,467-bp open reading frame (Fig. 1B) and shared the same 3′-noncoding region with variant 1. The first methionine in variant 2 was identified by the preceding stop codon (Fig. 1B). Genomic DNA analysis demonstrated that the two variants are the result of different splicing of the first exon (Fig. 1B). On the basis of the length of the open reading frame, we named variants 1 and 2 the AMPKγ3 short form (AMPKγ3S; AF525501) and the AMPKγ3 long form (AMPKγ3L; AF525500), respectively. The Mouse Genome Informatics ID for both γ3S and γ3L is MGI:1891343. γ3S contains 465 deduced amino acids, whereas γ3L contains 490 amino acids. The two splice isoforms share 100% identity except for an extra 25 amino acids at the NH2-terminal of γ3L (Fig. 1B). Both AMPKγ3S and AMPKγ3L share high identity and similar overall protein structure with pig and human AMPKγ3 subunits, and little identity with the γ1 and γ2 subunits.

Figure 1C shows the deduced amino acid sequence alignment of mouse γ3S and γ3L compared with pig γ3, the two different human γ3 sequences that were reported with different initiation sites, and rat γ3 (XM_237293). Mouse γ3S shows overall 85.5% identity to human (AF214519) and pig γ3. Mouse γ3L is most similar to another reported human γ3 gene (NM_017431) that contains an altered initiation site and also has 85.5% identity. The four CBS domains are highly conserved, showing 96% identity in all species, whereas the remainder of the NH2-terminal region shows 66% identity. Interestingly, both AMPKγ3 splice variants are rich in serine and threonine at the NH2-terminal region; these are potential phosphorylation sites for a number of protein kinases including glycogen synthase kinase 3, protein kinase A, and protein kinase C, based on consensus sequence searches for these kinases using PhosphoBase (http://www.cbs.dtu.dk/databases/PhosphoBase/).

AMPKγ3 protein expression. To determine the expression pattern of AMPKγ3, we generated an antibody against a conserved sequence within COOH terminus of mouse γ3S and γ3L, human γ3, and pig γ3 (Fig. 1C). Immunoblotting with γ3 antibody revealed that AMPKγ3 is highly expressed in gastrocnemius muscle, with no detectable signal in heart, brain, lung, liver, kidney, pancreas, spleen, white fat, or brown fat (Fig. 2A). These results are consistent with Northern hybridization data from human tissues, which showed that AMPKγ3 is specifically expressed in skeletal muscle (11, 31). Interestingly, white fast-twitch glycolytic skeletal muscle that contains mostly type IIb fibers (white quadriceps and white gastrocnemius) showed higher levels of AMPKγ3 expression compared with the red fast-twitch oxidative-glycolytic muscle, which contains primarily type IIa fibers (red quadriceps and red gastrocnemius). AMPKγ3 was not detectable in soleus, a muscle that is composed almost exclusively of red slow-twitch oxidative type I fibers. This expression pattern suggests that AMPKγ3 plays a unique role in skeletal muscle, especially in the more fast-twitch glycolytic fiber types.
overexpressed γ3S suggests that the expressed protein in the cells was partially degraded.

In addition, γ3L is more abundant compared with γ3S at the mRNA level (Fig. 2C), and this is consistent with Western blotting data showing that γ3L is the predominant form in skeletal muscle (Fig. 2B). The γ3S variant was detected by PCR (5'-RACE), but protein was not expressed. This may be explained by the following possibilities: 1) the lower γ3S
mRNA results in very low levels of γ3S protein, beyond the detection of the γ3 antibody; 2) there may be an inhibitory mechanism at the translational or posttranslational steps that suppresses γ3S protein expression; and/or 3) γ3S protein is less stable and is more rapidly degraded.

The molecular masses of γ3S and γ3L in SDS-PAGE were 64 and 67 kDa, respectively, different from their predicted molecular masses of 51 and 54 kDa. The reason for the mobility shift of γ3 in a denatured gel is unclear, although interestingly, the same phenomenon has been observed in the AMPKβ1 subunit. In SDS-PAGE the molecular mass of AMPKβ1 isolated from rat liver is 38 kDa higher than the predicted molecular mass of 30 kDa (50).

Association of γ3 subunit with α- and β-subunits. To determine whether the γ3 subunit associates with the α catalytic subunit, we used mouse muscle lysates for the immunoprecipitation of AMPKα, using an anti-AMPKα-specific antibody that recognizes both α1 and α2. As shown in Fig. 3A, the anti-AMPKα1/2 antibody immunoprecipitates α1/2 and coprecipitates both the β1 subunit and the γ3 subunit from mouse skeletal muscle lysates. There was also concomitant immunodepletion of AMPKα1/2, AMPKβ1, and the AMPKγ3 subunits from the immunosupernatant. In contrast, control IgG did not pull down any of these proteins, and AMPKα1/2, AMPKβ1, and AMPKγ3 are preserved in the supernatant. This experiment also confirms that AMPKγ3 runs as a 67-kDa band in SDS-PAGE (Fig. 2, A and B, and Fig. 3A). When AMPKβ1 was immunoprecipitated in skeletal muscle lysates with the use of a commercially available antibody, AMPKβ1 was barely detectable (not shown), and because an antibody for the immunoprecipitation of AMPKβ2 was not commercially available, the potential association of β with γ3 was determined by expressing exogenous AMPKβ1 and γ3L in L6 myoblast cells. We chose γ3L because this is the endogenous splice isoform of AMPKγ3.

Fig. 1. A: nucleotide sequence and the deduced amino acid sequence of AMP-activated protein kinase (AMPK) γ3S. Nucleotides and amino acids are numbered on the left and right, respectively. The translation initiation codon is used as the starting nucleotides for numbering. The in-frame translation termination codon is marked by an asterisk. B: AMPKγ3S and AMPKγ3L are different splice isoforms. Top: representation of AMPKγ3S, the translation initiation codon is located at exon III. Bottom: representation of AMPKγ3L, the translation initiation codon is localized in exon I. The nucleotide sequence and the deduced amino acid sequence specific for AMPKγ3L are shown in boxes. The intron boundaries between exons I and II are indicated in parenthesis. The in-frame stop codon is indicated by an asterisk. C: comparison of the mouse AMPKγ3 amino acid sequence with human, pig, and rat sequences. Alignment was performed between amino acid sequences of mouse γ3S (My3S; AF525501), mouse γ3L (My3L; AF525500), human γ3 (Hy3; AJ249977 and AF214519), pig γ3 (Pγ3; AF214520), and rat γ3 (Rγ3; XM_237293). AMPKγ3S amino acid sequence was used as a master sequence. Dash indicates identity to the master sequence; dot indicates alignment gaps. Cystathionine β-synthase (CBS) domain sequences are boxed, and the peptide sequence (γ3L 457–467) in bold letters was used to generate the AMPKγ3 antibody.

Fig. 2. A: distribution of AMPKγ3 in different mouse tissues. Protein (20 μg) from different mouse tissue lysates was separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. AMPKγ3 was detected by immunoblotting with an anti-AMPKγ3-specific antibody. B: AMPKγ3 splice isoforms in mouse and human skeletal muscles. Mouse and human skeletal muscle lysates with endogenous AMPKγ3 and cell lysates of L6 myoblasts with overexpressed (OE) AMPKγ3L and AMPKγ3S were separated by 8% SDS-PAGE and immunoblotted with an anti-AMPKγ3 antibody. C: comparison of the mRNA levels of γ3L and γ3S in mouse skeletal muscle. PCR products from sense adaptor primer attached to double-strand cDNA including both γ3L and γ3S and an antisense primer conserved in both γ3L and γ3S (see METHODS) were separated by 1% agarose gel. DNA sequence analysis demonstrated that the top band is from γ3S and the bottom band is from γ3L. Because the 5′-noncoding region plus coding region of γ3S PCR product is longer than that of γ3L, γ3S migrates more slowly through the gel.
AMPKγ3 in mouse skeletal muscle. This experiment revealed that expressed AMPKγ3 could be coimmunoprecipitated with the β1 subunit (Fig. 3B).

Overexpression of AMPKγ3S and AMPKγ3L in mouse TA muscle in vivo. To determine the effects of γ3S and γ3L on AMPK activity and glycogen metabolism in skeletal muscle, we used a gene transfer/electroporation system to express the two splice variants in mouse TA muscle in vivo. By using this method in preliminary experiments using the LacZ gene, 85.7 ± 2.3% (n = 6) of fibers were determined to express β-galactosidase (18). AMPKγ3S and AMPKγ3L expression levels were measured at days 7, 9, and 14 after gene transfer/electroporation, and maximal expression occurred at day 9 (∼1.5- to 2.5-fold greater than endogenous AMPKγ3); thus all subsequent experiments were done using this time point. Figure 4A, top, shows that, as predicted, Flag-tagged AMPKγ3L ran at a higher molecular mass compared with Flag-tagged γ3S. Figure 4A, middle, shows the degree of overexpression of γ3S and γ3L compared with endogenous γ3 and also confirms the experimental results shown in Fig. 2B, demonstrating that γ3L splice variant is endogenously expressed in mouse skeletal muscle. Figure 4A, bottom, shows that recombinant AMPK γ3S and AMPKγ3L did not affect endogenous γ1 protein levels, suggesting that γ3 overexpression does not replace this isoform. Endogenous γ2 isoform was not determined because anti-γ2 antibodies were not commercially available. In lysates from TA muscle, overexpressed AMPKγ3S and AMPKγ3L could be coimmunoprecipitated with the α-subunit (Fig. 4B).

Overexpression of both AMPKγ3S and AMPKγ3L in vivo resulted in a significant increase in AMPKα1 catalytic activity (Fig. 5A, left). The increase in AMPKα1 activity was associated with an increase in AMPKα1 Thr172 phosphorylation, determined by AMPKα1 immunoprecipitation followed by Western blotting using a phosphospecific AMPKα antibody (Fig. 5A, right). The increases in AMPKα1 activity and phosphorylation were not due to an increase in AMPKα1 protein expression (Fig. 5A, right). In contrast, AMPKγ3S and AMPKγ3L expression in vivo had no effect on AMPKα2 activity, phosphorylation, and expression (Fig. 5B), although the phosphorylation of AMPKα2 in the basal condition was barely detectable. The increase in AMPKα1 activity was not associated with changes in glycogen concentrations in the muscle (Fig. 5C). Consistent with this finding, overexpression of AMPKγ3S and AMPKγ3L had no effect on glycogen synthase protein expression or the phosphorylation state of glycogen synthase (Fig. 5C). Several other muscle proteins were also not altered by expression of AMPKγ3S and AMPKγ3L, including GLUT-4, citrate synthase, and phosphoacetyl CoA carboxylase proteins known to be involved in the regulation of glucose uptake, mitochondria biogenesis, and fatty acid oxidation, respectively (data not shown).

In addition, AMPKγ3L or AMPKγ3S overexpression did not change the susceptibility of AMPK to activation by con-
DISCUSSION

We cloned the mouse AMPKγ3 gene and determined that there are two translation initiation sites that lead to two different AMPKγ3 splice isoforms, a short form, γ3S, and a long form, γ3L (Fig. 1, A–C). Both γ3L and γ3S are full-length genes (Fig. 1B) and, by genomic DNA analysis, are identified as two splice isoforms (Fig. 1B). Western blot analysis of mouse and human muscle lysates suggests that γ3L is the predominant form in mouse skeletal muscle (Fig. 2B), whereas γ3S is probably the predominant form in human skeletal muscle (Fig. 5). The finding suggests that both splice variants are physiologically relevant. This finding also clarifies discrepancies in previous reports showing differences in translation initiation sites for the human γ3 gene (11, 31). By immunoprecipitation with anti-α- and β-subunit antibodies, our data also show that the γ3 subunit is associated with the α- and β-subunits (Fig. 3, A and B). Thus the γ3 subunit can be a component of the AMPK heterotrimeric complexes, similar to what has been demonstrated for the γ1 subunit (50) and the yeast isoform of the γ-subunit, SNF4 (9).

Western blot analysis of mouse tissue lysates using a γ3-specific antibody clearly showed that AMPKγ3 was only detected in skeletal muscle. However, another report has suggested that γ3 is mainly distributed in brain and testis, by measuring the proportion of total AMPK activity (11). Although we do not have an explanation for the difference between our work and that of Cheung et al. (11), our data are consistent with Northern blotting results from two independent groups showing γ3 mRNA only in skeletal muscle (11, 31). Though γ3 is only present in skeletal muscle, we found different levels of expression of γ3 in muscle composed of various muscle fiber types. White muscles, which contain predominantly fast-twitch glycolytic (type IIb) fibers showed the highest levels of γ3 expression. The red muscles, which contain predominantly fast-twitch oxidative glycolytic (type IIa) fibers, had lower levels of expression, whereas γ3 was undetectable in soleus muscle, which is a slow-twitch oxidative muscle with predominantly type I fibers. The type IIb fibers present predominantly in white muscles are abundant in fast-twitch myosin isoforms and have a more glycolytic metabolic phenotype. These fibers are recruited during high-intensity...
types of contractions, rapidly providing energy through glycolysis. Whether wild-type γ3 regulates AMPK activity in a manner that promotes the rapid utilization of glycogen is not known, but this is an interesting hypothesis to test in future studies. The lower levels of γ3 expression in the red oxidative fibers provide an explanation for the previous observation that excess glycogen accumulation specifically occurs in white muscles in pigs with the RN− mutation in the PRKAG3 gene (R200Q mutation in AMPKγ3) (17). Interestingly, incubation of muscles with the AMPK activator 5-aminomimidazole-4-carboxamide ribonucleoside (AICAR) only increases AMPK activity in the highly glycolytic white epitrochlearis muscle but has no effect in the red oxidative (type I) soleus muscle (1, 27), (Hirshman MF, Goodyear LJ, and Hayashi T, unpublished observation). It is not known whether γ3 expression is needed for AMPK to respond to AICAR in mouse skeletal muscle.

We successfully overexpressed AMPKγ3S and AMPKγ3L in mouse skeletal muscle by using a gene transfer and electroporation system and found that both splice variants associate with the endogenous AMPKα protein (Fig. 4B). Using this experimental system, we found that expression of AMPKγ3S and AMPKγ3L increased AMPKα activity and phosphorylation but had no effect on AMPKα2 activity and phosphorylation. The increase in AMPKα1 activity was not due to an increase in AMPKα1 protein, suggesting that the increase in phosphorylation of AMPKα1 is responsible for the increase in AMPKα1 activity. The mechanism by which γ3 overexpression increases α1 phosphorylation and activity in vivo is unclear. Different γ-subunits have been reported to account for different proportions of total AMPK activity (11), and therefore an exchange between isoforms could lead to a change in AMPK activity. However, endogenous γ1 expression was not changed in response to γ3 overexpression (Fig. 4A), making this mechanism unlikely to account for the increase in AMPKα1 activity. Another possibility is that γ3 could favorably alter the conformation of α1-containing AMPK heterotrimers, which in turn could enhance α1 phosphorylation by upstream signals such as AMPK kinase or retard dephosphorylation by phosphatases. Unfortunately, we are not able to directly compare the amount of γ3 subunit associated with α1 and α2, because anti-AMPKα1 and anti-AMPKα2 antibodies have different affinities to α1 and α2 subunits.

There are now multiple examples demonstrating α1- and α2-specific AMPK regulation in response to various perturbations (6, 27, 45). One example is in cardiac hypertrophy, where AMPKα1 activity and expression are increased whereas α2 expression is decreased (45). Exercise training also specifically upregulates AMPKα1 in skeletal muscle (6). Another example is the recent report that obesity-related insulin resistance is associated with a specific impairment in contraction-stimulated α1 activity in skeletal muscle in rats (6). Because we found that γ3 overexpression results in an α1-specific activation, in future studies it will be interesting to determine whether altered γ3 expression plays a role in the impaired α1 response observed in these insulin resistant animals.

The AMPKγ3 mutation from arginine to glutamine at position 200 (R200Q) results in muscle glycogen accumulation in the Hampshire pig. In contrast, the valine-to-isoleucine mutation at position 199 in the same species reduces muscle glycogen content (3, 12). The fact that adjacent mutations at V199 and R200 in the CBS domain of the γ3 subunit cause opposite effects on muscle glycogen content suggests that structural changes in this part of the CBS domain alter enzyme function. In our study, modest overexpression of wild-type AMPKγ3 did not change muscle glycogen concentrations and had no effect on the expression and phosphorylation of glycogen synthase (Fig. 5C), despite significant increases in AMPKα1 activity. Furthermore, despite major differences in the amount of endogenous γ3 protein among muscles of different fiber types, we did not observe a significant difference in muscle glycogen concentrations in these various muscles (Yu H, Hirshman MF, and Goodyear LJ, unpublished observation). These results suggest that wild-type AMPKγ3 does not directly regulate muscle glycogen concentrations and that the mutations are the cause of the alterations in glycogen metabolism.

In summary, we have cloned the full-length mouse AMPKγ3 gene and identified two γ3 splice variants, γ3S and γ3L. Both γ3S and γ3L can associate with the α- and β-subunits of AMPK. These two splice variants appear to be differentially expressed in human and mouse skeletal muscle. Overexpression of either γ3S or γ3L results in a specific increase in AMPKα1 activity and phosphorylation, but these changes are not associated with alterations in muscle glycogen.

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