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Characterization of the role of γ2 R531G mutation in AMP-activated protein kinase in cardiac hypertrophy and Wolff-Parkinson-White syndrome

Joanna K. Davies,[1] Dominic J. Wells,[2] Ke Liu,[2] Helen R. Whitrow,[1] Tyrone D. Daniel,[1] Robert Grignani,[3] Craig A. Lygate,[3] Jürgen E. Schneider,[3] Gaëtane Noël,[4] Hugh Watkins,[3] and David Carling[1]

[1]*Cellular Stress Group, Medical Research Council Clinical Sciences Centre, Hammersmith Campus, Imperial College London;* [2]*Gene Targeting Unit Department of Cellular and Molecular Neuroscience, Division of Neuroscience and Mental Health, Charing Cross Campus, Imperial College London, London;* [3]*Department of Cardiovascular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom; and* [4]*Laboratory of Physiological Chemistry, Christian de Duve Institute of Cellular Pathology, Brussels, Belgium*

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Davies, Joanna K., Dominic J. Wells, Ke Liu, Helen R. Whitrow, Tyrone D. Daniel, Robert Grignani, Craig A. Lygate, Jürgen E. Schneider, Gaëtane Noël, Hugh Watkins, and David Carling. Characterization of the role of γ2 R531G mutation in AMP-activated protein kinase in cardiac hypertrophy and Wolff-Parkinson-White syndrome. *Am J Physiol Heart Circ Physiol* 290: H1942–H1951, 2006. First published December 9, 2005; doi:10.1152/ajpheart.01020.2005.—AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that plays a key role in the regulation of energy metabolism. In humans, mutations in the γ2-subunit of AMPK cause cardiac hypertrophy associated with Wolff-Parkinson-White syndrome, characterized by ventricular preexcitation. The effect of these mutations on AMPK activity and in development of the disease is enigmatic. Here we report that transgenic mice with cardiac-specific expression of γ2 harboring a mutation of arginine residue 531 to glycine (RG-TG) develop a striking cardiac phenotype by 4 wk of age, including hypertrophy, impaired contractile function, electrical conduction abnormalities, and marked glycogen accumulation. At this stage, AMPK activity isolated from hearts of RG-TG mice was almost completely abolished but could be restored after phosphorylation by an upstream AMPK kinase. At 1 wk of age, there was no detectable evidence of a cardiac phenotype, and AMPK activity in RG-TG hearts was similar to that in nontransgenic, control mice. We propose that mutations in γ2 lead to suppression of total cardiac AMPK activity secondary to increased glycogen accumulation. The subsequent decrease in AMPK activity provides a mechanism that may explain the development of cardiac hypertrophy in this model.

glycogen metabolism; heart energetics; signal transduction pathways

AMP-ACTIVATED PROTEIN KINASE (AMPK) is the downstream component of a protein kinase cascade that is conserved in all eukaryotic species examined to date (5, 14, 15). AMPK plays a key role in the regulation of energy homeostasis and is activated by a rise in the AMP-to-ATP ratio within the cell, such as occurs after ATP depletion (5, 14, 15). In the heart, AMPK is activated during ischemia, leading to phosphorylation and inhibition of acetyl-CoA carboxylase (20) and phosphorylation and activation of phosphofructo-2-kinase (23). The overall effect of this is to increase the rates of fatty acid oxidation (20) and glycolysis (23).

AMPK is a heterotrimeric enzyme, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). The β-subunit contains a domain that is found in a number of enzymes that metabolize the α1–6 branch points in α1–4-linked glucans, such as glycogen and starch, and has subsequently been termed a glycogen-binding domain (18, 27). The γ-subunit contains four cystathionine-β-synthase (CBS) domains. These domains are found in a wide variety of proteins, although the function of the domains is incompletely understood. However, there is strong evidence to suggest that in AMPK the CBS domains are involved in the allosteric regulation of the kinase by adenine nucleotides.

Three isoforms of the γ-subunit have been identified in mammals, each encoded by a separate gene (5). In rats, the γ1 and γ2 isoforms are widely expressed (6), whereas expression of the γ3 isoform appears to be restricted to skeletal muscle (22). Mutations in the gene encoding the γ2-subunit (*PRKAG2*) cause cardiac hypertrophy with associated Wolff-Parkinson-White (WPW) syndrome in humans (1, 3, 11). The effect of these mutations on AMPK activity is controversial (5). AMP stimulation was reported to be significantly reduced by three mutations (R302Q, H383R, and R531G) after transient expression of γ2-containing AMPK complexes in mammalian cells, whereas a mutation that results in the insertion of a leucine residue in the linker region between CBS domain 1 and CBS domain 2 had no significant effect on AMPK activity (8). In contrast, a separate study (2) reported increased AMPK activity in hearts from transgenic mice expressing γ2 harboring a N488I mutation relative to either wild-type γ2 (γ2WT) transgenic mice or nontransgenic mice.

We report here the effect of cardiac-specific expression of γ2WT and a mutant form of γ2 harboring a missense mutation of arginine residue 531 to glycine (R531G). By 4 wk of age, transgenic mice expressing mutant γ2 (RG-TG) develop a marked cardiac phenotype that closely mimics the human disease, including left ventricular hypertrophy, impaired cardiac function, ventricular preexcitation, and massive glycogen accumulation. Furthermore, RG-TG mice had significantly increased mortality. After onset of the cardiac phenotype, total, as well as γ2-associated, AMPK activity in hearts from RG-TG mice was almost completely abolished. Importantly, AMPK activity was increased after phosphorylation by an upstream AMPK kinase in vitro. At 1 wk of age, we were unable to distinguish any significant phenotype, and at this point, AMPK

Address for reprint requests and other correspondence: D. Carling, Cellular Stress Group, MRC Clinical Sciences Centre, Hammersmith Campus, Imperial College London, DuCane Rd., London W12 ONN, UK (e-mail: dcarling@imperial.ac.uk).

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activity was not significantly different in hearts from nontransgenic, wild-type transgenic (WT-TG), or RG-TG mice. Our findings demonstrate that the phenotype in RG-TG mice is a direct consequence of the mutation in γ2 but that the underlying cause of the phenotype resulting from the γ2 mutation may be unrelated to a direct effect of the mutation on AMPK activity. The decrease in total AMPK activity in hearts may be a consequence of the increased glycogen accumulation, which in turn would lead to changes in the regulation of downstream targets of AMPK. This hypothesis provides a model that may explain the cardiac hypertrophy associated with the disease.

MATERIALS AND METHODS

Transgene construct. Human cDNAs encoding wild-type and R531G AMPK γ2 (8) were cloned into a vector containing the α-myosin heavy chain (α-MHC) promoter, human growth hormone 3′-untranslated region, and a polyA signal sequence. The presence and orientation of the inserted cDNAs were confirmed by DNA sequence analysis. Digestion with *Bam*HI was used to linearize the transgene, which was subsequently purified using a Qiagen DNA purification kit. Transgenic mice were generated by microinjection of the γ2 transgene into fertilized mouse oocytes. The founders were produced on a hybrid background of C57BI10 × CBA/Ca. Transgenic animals were bred back to F1 hybrids, thus keeping the background mixed. All animals procedures were carried out under license approved by the 1986 British Home Office Animals Scientific Procedures Act and were approved by an Institutional Committee.

Genotyping. Transgenic founders were identified using PCR amplification of tail DNA purified by phenol/chloroform extraction. Primers used for genotyping were 5′-AGAATCGCATCTATGCCTCG-3′ (forward) and 5′-CTCTCCCACAGTGGCGC-3′ (reverse). These primers span an intron in *PRKAG2* and so, therefore, under the PCR conditions used, will only detect a product when the transgene is present. The presence of a 360-bp PCR product confirmed the insertion of the transgene into the genomic DNA. F1 mice and subsequent generations of transgenic mice were screened using the same PCR protocol but using DNA present in an ear biopsy.

Real-time PCR. Transgene copy number was calculated by using SYBR Green real-time PCR. Briefly, PCR was carried out in triplicate on salt-purified ear clip DNA and plasmid standards with the use of the QuantiTect SYBR Green PCR Kit (Qiagen) on the Rotor-Gene (Corbett Research). PCR was performed with γ2-specific primers chosen to span an intron, CCAGACACTCCCATCATCAAAGC (forward) and CTGCTGGTGTGAGGATCAGGGCTTG (reverse). Data were analyzed with Rotor-Gene software version 5.0 (Corbett Research).

Magnetic resonance imaging and cardiac function. Cine-MRI analysis was performed as reported previously (29).

Glycogen measurements. Cardiac glycogen content was determined as described previously (21). Briefly, glycogen was solubilized by using 1 M KOH and digested with amyloglucosidase to release glucose. After incubation with ATP in the presence of hexokinase, glucose-6-phosphate concentration was determined spectrophotometrically by measuring the production of NADPH at 340 nm in the presence of glucose-6-phosphate dehydrogenase. Glycogen content is shown as micrograms per milligram wet weight heart tissue. The degree of glycogen branching was measured spectrophotometrically as previously described (4).

Histology. Mouse hearts were excised, mounted, and rapidly frozen. Transverse sections of 10 μm were taken through the ventricles. Sections were fixed with the use of Bouin's solution (Sigma) and then stained with hematoxylin and eosin (Sigma). Glycogen was detected with periodic acid Schiff (PAS) staining (Sigma).

Electrocardiography. ECGs from conscious, unrestrained female mice aged 9–11 mo ($n = 2$, non-TG control; $n = 3$, WT-TG; $n = 4$, mutant transgenic) were recorded by using the AnonyMOUSE ECG screening tool (Mouse Specifics, Boston, MA) (7). Mice were allowed to acclimatize for at least 10 min after being handled before data were recorded.

AMPK activity determination. Hearts were homogenized (in mM) in 50 Tris·HCl (pH 7.5), 250 mannitol, 50 NaF, 5 sodium pyrophosphate, 1 EDTA, 1 dithiothreitol, and 0.1 phenylmethylsulfonyl fluoride. Insoluble material was removed by centrifugation and AMPK isolated by immunoprecipitation from the lysate (equivalent to 50 mg wet wt tissue) with the use of AMPK subunit-specific antibodies prebound to either protein A-Sepharose (rabbit antibodies) or protein G-Sepharose (sheep antibodies). Antibodies used in this study [sheep anti-α1, sheep anti-α2, sheep anti-γ1, sheep anti-γ2 (6), and a rabbit antibody that recognizes both β1 and β2 (pan-β) (33)] have been described previously. After immune complexes were extensively washed, AMPK activity was determined in the immune complexes by the incorporation of ^{32}P-radiolabeled phosphate into the synthetic SAMS peptide (HMRSAMSGLHLVKRR) (9) in the presence and absence of 0.2 mM AMP. Activities are calculated as micromoles of phosphate incorporated per minute per gram wet weight and are corrected for nonspecific activity by subtracting the incorporation measured in a control reaction using preimmune serum in the immunoprecipitation step.

In vitro phosphorylation of AMPK. AMPK was isolated by immunoprecipitation and the resulting immune complex incubated with 0.2 mM ATP and 5 mM $MgCl_2$ in the presence or absence of bacterially expressed Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK-β; 50 μg/ml) for 30 min at 30°C. After immune complexes were extensively washed, AMPK activity in the immune complex was determined by using the SAMS peptide assay.

Western blot analysis. Tissue lysates (50 μg) were resolved on 12% SDS-PAGE, transferred to a polyvinylidene membrane, and probed with an appropriate primary antibody, followed by either goat anti-rabbit or donkey anti-sheep antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology). Proteins were visualized by enhanced chemiluminescence (Pierce).

Statistical analysis. Results are plotted as means ± SE, and significance was determined by using an unpaired Student's *t*-test. A value of $P < 0.05$ was considered significant.

RESULTS

Generation of transgenic lines expressing γ2 AMPK. Wild-type human cDNA encoding full-length AMPK-γ2 and cDNA harboring a point mutation altering arginine 531 to glycine (R531G) were used as transgenes and were under the control of the cardiac-specific α-MHC promoter (13). The presence of the transgene was detected by PCR (see MATERIALS AND METHODS). Two founder lines each of γ2 wild-type (WT-TG2 and WT-TG12) and γ2 R531G (RG-TG16 and RG-TG22) transgenic mice were produced. Real-time PCR revealed that the transgene copy number varied between 12 and 70 in the different lines (data not shown). Despite this variation, Western blot analysis of heart lysates demonstrated that expression of γ2 protein was similar in all the lines (Fig. 1*A*) and was restricted to the heart (Fig. 1*B*). Under these conditions, we did not detect endogenous γ2, presumably due to the low level of expression of this isoform (6). In this study, we report results for the WT-TG2 and RG-TG22 lines.

RG-TG mice have a severe cardiac phenotype. Initial inspection of the mice revealed a dramatic increase in heart size in the RG-TG mice compared with WT-TG or non-TG mice. We used cine-MRI to study cardiac function in the transgenic animals. Figure 2, *A* and *B*, shows MRI scans of hearts from an adult non-TG mouse and an adult RG-TG mouse, respectively.

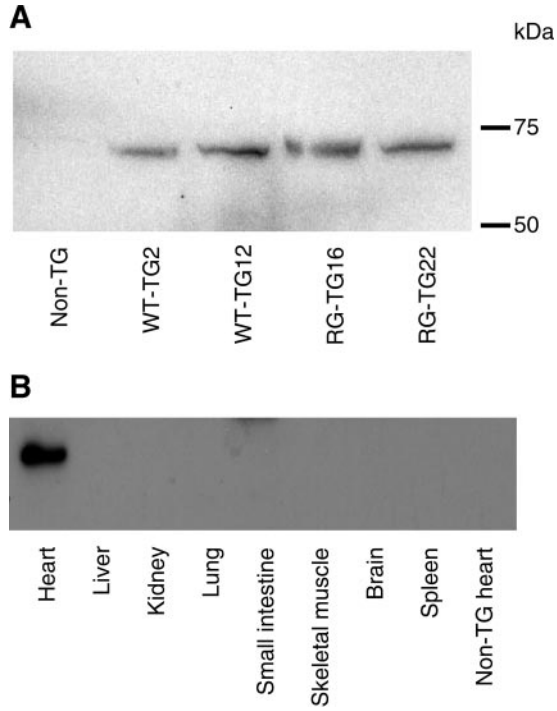

Fig. 1. Cardiac-specific expression of γ2 transgene. *A*: heart lysates (50 μg) from nontransgenic (non-TG), wild-type transgenic (WT-TG), and R531G transgenic (RG-TG) mice aged 8–12 wk subjected to Western blot analysis with a γ2-specific antibody. *B*: Western blot analysis of lysates (50 μg) from various tissues isolated from RG-TG mouse. In each case, a representative blot is shown.

Heart weight in RG-TG mice was dramatically increased relative to the non-TG heart from 4 wk of age (Fig. 2*C*). There was an approximately threefold increase in the ratio of heart weight to body weight in the RG-TG mice compared with either WT-TG or non-TG mice. A small but significant increase in this ratio was also evident in WT-TG relative to non-TG mice. RG-TG mice exhibited increased spontaneous mortality, and this was more pronounced in male mice (Fig. 2*D*). WT-TG showed no increase in mortality compared with non-TG littermates. Table 1 lists a number of parameters used to assess cardiac function. As can be seen, hearts from RG-TG mice had severely impaired cardiac function, with hypertrophy, dilation, and poor contractile function. These findings are typical of the human cardiac phenotype associated with γ2 mutations (3).

In humans, the γ2 mutations, including R531G, are also associated with ventricular preexcitation (12). Figure 3 shows the results of ECG analysis of hearts from non-TG mice compared with WT-TG and RG-TG mice. There was a significant shortening of the PR interval in hearts from RG-TG (14.6 ± 3.5 ms; $n = 4$) mice compared with hearts from either WT-TG (27.9 ± 1.4 ms; $n = 3$) and non-TG mice (30.1 ms; average of 2 values). In addition, QRS widening in the RG-TG heart was evident as indicated by an interval of 15.2 ± 1.3 ms ($n = 4$) in the RG-TG compared with 10.7 ± 1.4 m ($n = 3$) in WT-TG and 10.3 ms (average of 2 values) in non-TG. The electrical conduction abnormalities in the RG-TG mice are indicative of ventricular preexcitation similar to that observed in WPW syndrome.

Hearts from RG-TG mice have marked glycogen accumulation. Earlier studies have reported that a missense mutation resulting in substitution of asparagine 488 to isoleucine (N488I) causes a marked accumulation of cardiac glycogen (1, 2). Figure 4*A* shows that RG-TG hearts had striking accumulation of glycogen compared with either WT-TG or non-TG hearts. At 4 wk of age, glycogen levels in the RG-TG hearts were nearly 40-fold higher than in hearts from their non-TG littermates and nearly 20-fold higher than in WT-TG hearts. By 8 wk, cardiac glycogen levels were more than 80-fold higher than non-TG levels and 15-fold higher than WT-TG levels. These differences were still evident at 20 wk (Fig. 4*A*). There was also a modest but significant increase in glycogen content in WT-TG hearts compared with non-TG hearts (twofold at 4, 16, and 20 wk and fivefold at 8 wk). PAS staining of transverse heart sections revealed that RG-TG hearts had glycogen accumulation throughout the heart, whereas there was no detectable difference in staining between sections from WT-TG and non-TG hearts (Fig. 4, *B–D*). Staining with hematoxylin and eosin revealed the presence of intracellular deposits together with large vacuoles in the RG-TG hearts, whereas sections from WT-TG hearts were indistinguishable from non-TG hearts (Fig. 4, *E–G*).

We determined the degree of branching of the glycogen in hearts from non-TG, WT-TG, and RG-TG mice by measuring the absorbance spectra of the glycogen in complex with iodine. The maximum absorbance of the glycogen/iodine complex increases as branching decreases. The glycogen present in RG-TG hearts had an absorbance peak at ~460 nm, whereas WT-TG and non-TG glycogen had a peak at 410 nm, indicating that the glycogen in the RG-TG hearts was less branched than in the non-TG and WT-TG hearts. This pattern is similar to the spectrum found in Andersen's disease, another type of glycogenosis, where the predominant form of polysaccharide stored is polyglucosan, an amylopectin-like polymer (10, 25).

AMPK expression and activity in hearts. To characterize the effect of overexpression of human γ2 on the endogenous AMPK subunits, we performed Western blot analysis of heart extracts from 8-wk-old animals with antibodies against AMPK subunit isoforms (Fig. 5*A*). The levels of α2, β1, and β2 were increased in extracts from WT-TG and RG-TG hearts compared with non-TG hearts. We were unable to directly detect γ1 in heart extracts by Western blotting, and, therefore, to assess γ1 expression, we isolated AMPK from heart extracts by immunoprecipitation using γ1-specific antibodies and probed the immune complexes with a pan-β antibody (Fig. 5*B*). There was no obvious difference in the amount of β-subunit coprecipitated by the γ1 antibody, indicating that the level of AMPK-γ1 complex was not altered in hearts from γ2 transgenic mice.

We measured AMPK activity in immune complexes isolated by immunoprecipitation from heart extracts using isoform-specific AMPK antibodies. Remarkably, AMPK activity in hearts from either 4- or 8-wk-old RG-TG mice was almost undetectable in immune complexes isolated by using γ2-specific antibodies (Fig. 5*C*), despite the over-expression of γ2 in these hearts. There was also a slight decrease in AMPK-γ2 activity in WT-TG hearts compared with non-TG hearts. We next measured AMPK activity in 8-wk-old hearts in immune complexes isolated with α1-, α2-, or γ1-specific antibodies (Fig. 5*D*). In all cases, AMPK activity in RG-TG hearts was

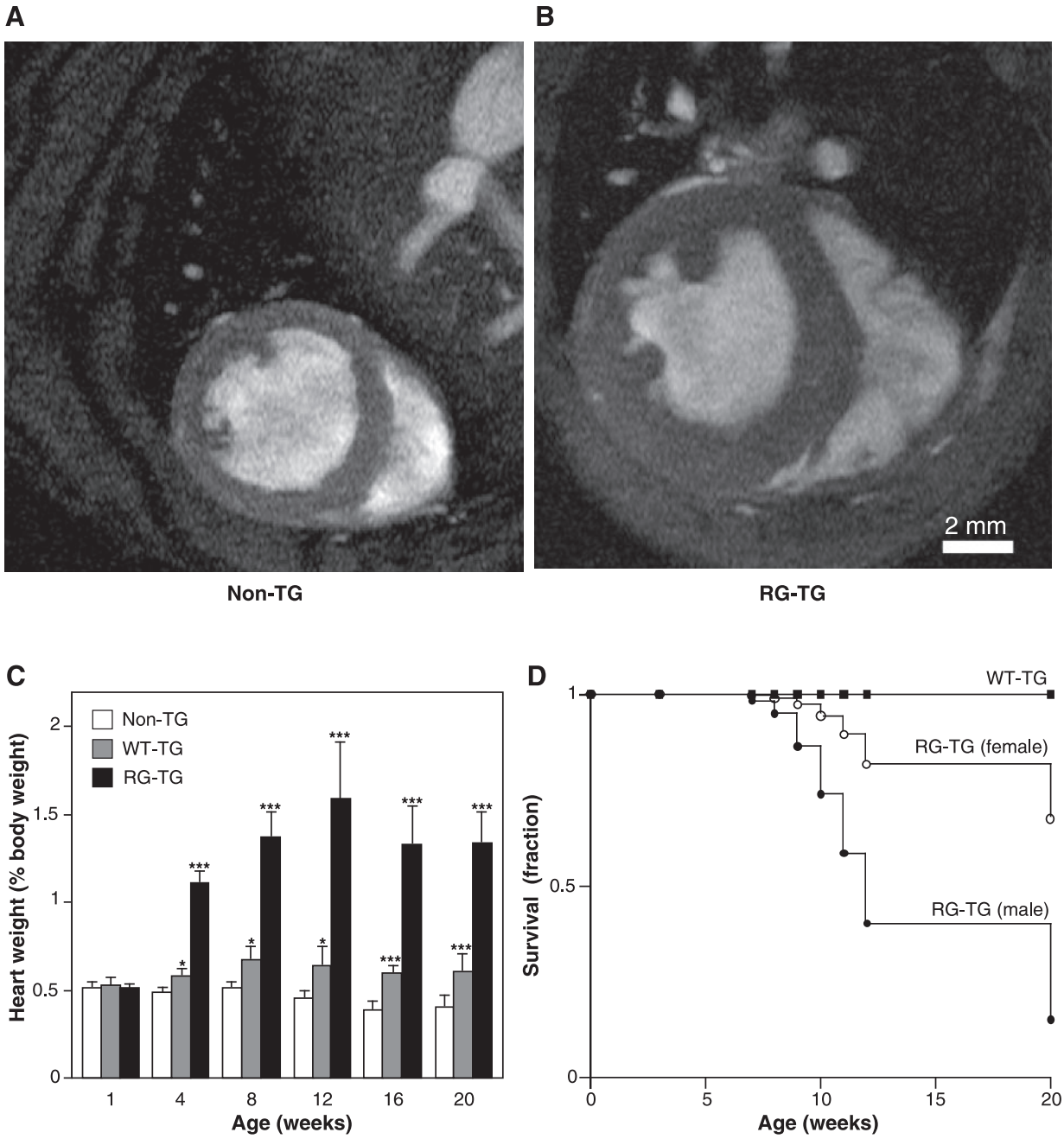

Fig. 2. Expression of γ2 R531G causes cardiac hypertrophy and increased mortality. Midventricular, end-diastolic cine-MRI frame in short-axis orientation is shown of a non-TG control (*A*) and a RG-TG (*B*) mouse, both ~6 mo of age. Note that the heart of the RG-TG mouse is strikingly dilated and hypertrophied. Scale bar for both images is 2 mm. *C*: heart weight as a percentage of body weight. In each case, results are means ± SE of 4–10 mice. Statistically significant differences are as follows: $*P < 0.05$ relative to non-TG mice; $***P < 0.005$ relative to non-TG or WT-TG mice. *D*: Kaplan-Meyer plot for WT-TG (■, combined data for males and females) and RG-TG female (○) and male (●) mice.

barely above that present in control immunoprecipitations measured in immune complexes isolated by using a preimmune serum. AMPK activity in WT-TG hearts was also markedly reduced, although it was significantly higher than in the RG-TG hearts. Similar results were obtained in hearts from older animals (data not shown). No AMPK activity was detectable in immune complexes isolated with γ3-specific antibodies (results not shown). We (8) previously reported that the R531G mutation abolishes AMP stimulation of the AMPK complex. AMP stimulated AMPK-γ2 activity 1.92 ± 0.13-fold ($n = 9$) and 1.95 ± 0.21-fold in non-TG and WT-TG hearts, respectively. However, we were unable to determine directly the AMP stimulation of AMPK-γ2 from the RG-TG mice because the activity was too low to measure reliably (see DISCUSSION).

Activation of AMPK requires phosphorylation of threonine-172 within the catalytic α-subunit by an upstream kinase (16). The level of T172 phosphorylation in the RG-TG extract was markedly diminished relative to non-TG hearts (Fig. 5*E*). T172 phosphorylation was also substantially reduced in extracts

Table 1. *Parameters for assessing cardiac function*

	Non-TG	WT-TG	RG-TG
n	5	5	4
Body weight, g	29±3	27±3	25±3
LV $mass_i \times 10^3$, g	3.2±0.2	4.2±0.3	8.0±0.5
End-diastolic $volume_i$, μl/g	2.1±0.3	2.1±0.1	3.7±0.5
End-systolic $volume_i$, μl/g	0.9±0.3	0.8±0.2	2.7±0.5
Ejection fraction, %	58±7	64±6	26±7
Stroke $volume_i$, μl/g	1.2±0.1	1.3±0.1	0.9±0.2
Heart rate, beats/min	494±44	454±36	424±33
Cardiac $output_i$, $\mu l \cdot min^{-1} \cdot g^{-1}$	0.60±0.05	0.60±0.06	0.40±0.09

Values are means ± SE; *n,* no. of mice. Subscript index i for left ventricular (LV) mass, end-diastolic volume, end-systolic volume, stroke volume, and cardiac output denotes normalization to body weight for these values. Differences between nontransgenic (non-TG) and R531G (RG-TG) and between wild-type transgenic (WT-TG) and R531G (RG-TG) mice were statistically significant ($P < 0.05$), except for heart rate and body weight. Between non-TG and WT-TG mice, a significant difference was only found for LV $mass_i$; $P < 0.01$.

from the WT-TG hearts. These findings are consistent with the decreased AMPK activity that we observed from heart extracts. To determine whether AMPK isolated from transgenic mouse hearts could be activated by phosphorylation, AMPK present in γ2-immune complexes was incubated with recombinant CaMKK-β, an upstream AMPK kinase (17, 19, 34), and MgATP. AMPK activity from both WT-TG and RG-TG hearts was increased significantly by this treatment (Fig. 5*F*). AMPK-γ2 activity from WT-TG hearts was stimulated a further twofold by AMP, whereas there was no effect of AMP on AMPK-γ2 activity from RG-TG hearts treated with CaMKK-β. AMPK activity in the absence of AMP was the same for complexes isolated from WT-TG or RG-TG mice. In a similar experiment, we isolated γ1-complexes and treated these with CaMKK-β (Fig. 5*G*). AMPK-γ1 activity in hearts from WT-TG and RG-TG was significantly increased after incubation with CaMKK-β (3.3-fold for WT-TG and 5.2-fold for RG-TG). In non-TG hearts, AMPK-γ1 activity was relatively high before treatment and was increased only slightly (1.3-fold) after incubation with CaMKK-β.

AMPK expression and activity in hearts from 7-day-old mice. The results detailed in *AMPK expression and activity in hearts* indicate that total AMPK activity is significantly reduced in hearts from WT-TG and RG-TG mice from 4 wk of age. A previous study (2), however, reported increased AMPK activity in hearts expressing γ2 harboring a mutation of asparagine 488 to isoleucine (N488I). In that study, AMPK activity was measured in 1-wk-old hearts. To determine whether the differences between our findings and those of the previous study might be due to the difference in the age of the animals, we measured AMPK subunit expression and activity in hearts from animals aged 1 wk. Although expression of γ2 was increased in both the WT-TG and RG-TG mice relative to non-TG mice, we did not detect any significant change in the expression of other AMPK subunits (Fig. 6*A*). AMPK activity measured in immune complexes isolated with α1-, α2-, γ1-, or γ2-specific antibodies was not significantly altered among non-TG, WT-TG, and RG-TG hearts (Fig. 6*B*). In addition, there was no significant difference in heart weight (Fig. 2*C*) or glycogen content (results not shown) between non-TG, WT-TG, and RG-TG hearts at this age.

DISCUSSION

In this study, we used transgenic mice with cardiac-specific expression of human AMPK-γ2 as a model system for understanding the molecular basis by which mutations in this protein cause cardiac hypertrophy and WPW syndrome in humans. Mice expressing γ2 harboring a mutation of arginine to glycine at residue 531 develop a phenotype that is remarkably similar to the human disease. RG-TG mice had marked cardiac hypertrophy, severely impaired cardiac function, electrical conduction abnormalities consistent with WPW syndrome, and striking accumulation of cardiac glycogen. In contrast, mice expressing γ2WT showed no evidence of impaired cardiac

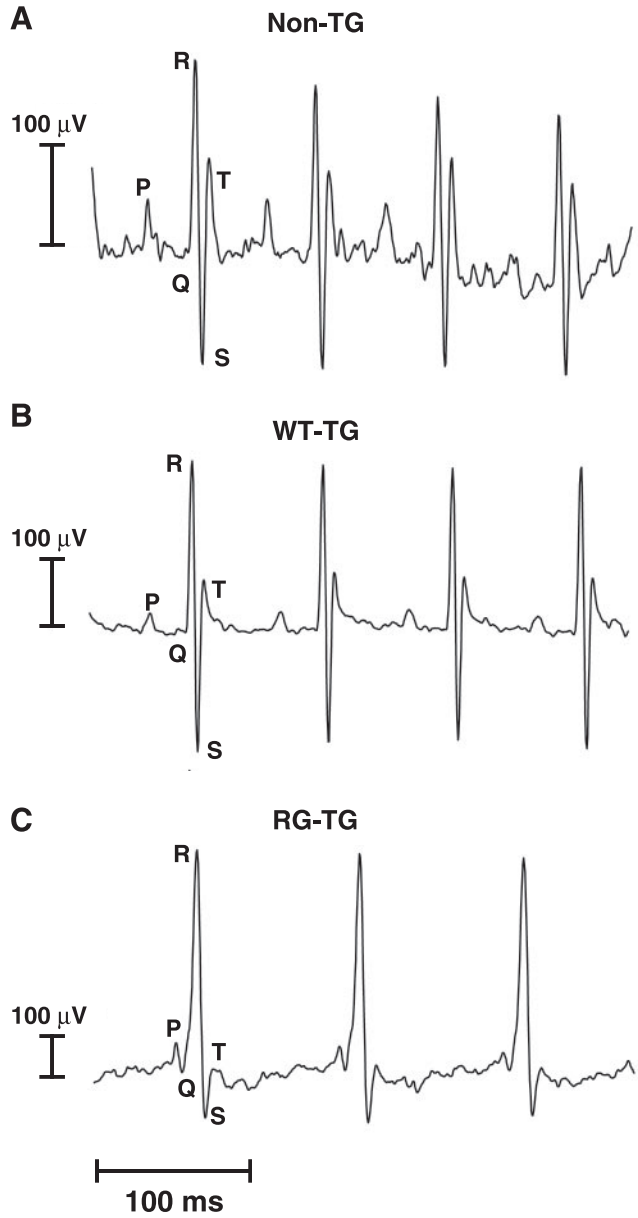

Fig. 3. Electrophysiological analyses. Representative surface ECGs from conscious, unrestrained adult (~20 wk old) mice are shown: non-TG (*A*), WT-TG (*B*), and RG-TG (*C*) animals. PR interval in RG-TG mice is significantly shorter than in either non-TG or WT-TG mice and is associated with a slurred upstroke and widening of the QRS complex, which is indicative of preexcitation.

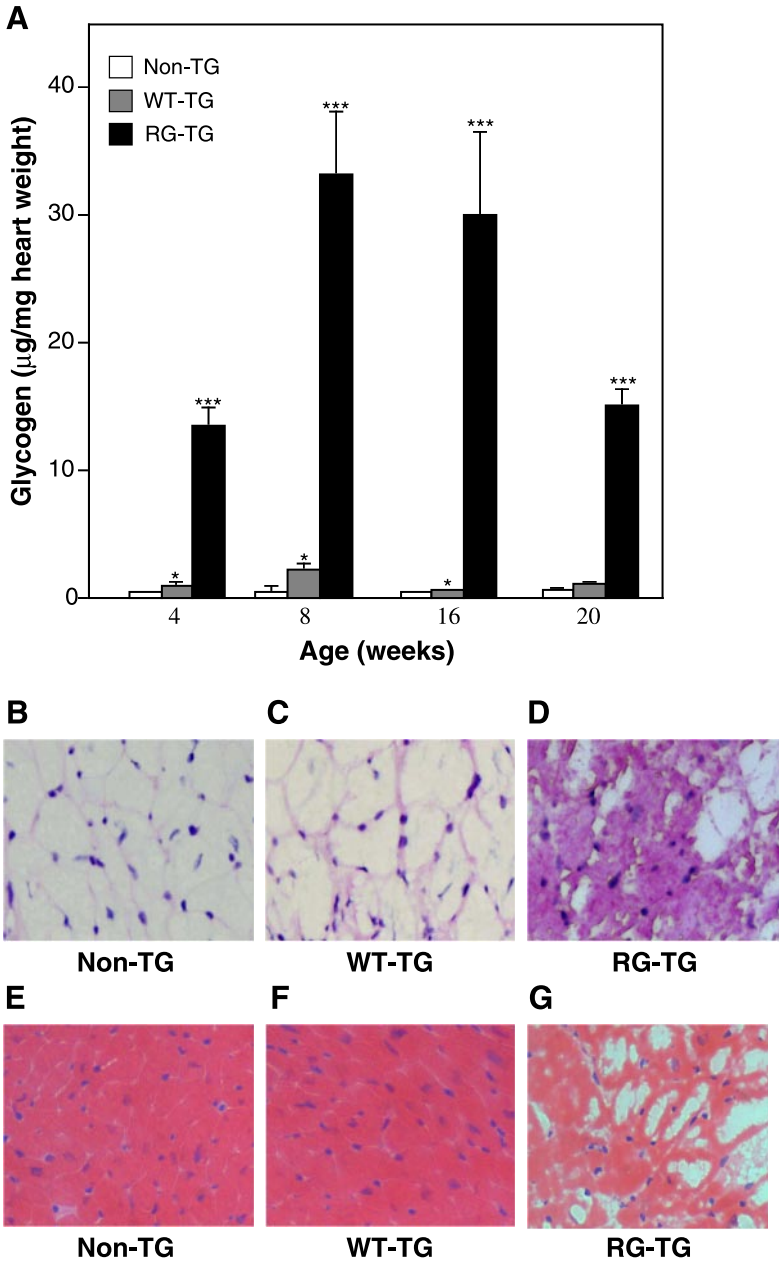

Fig. 4. Glycogen accumulation in hearts from γ2 R531G transgenic mice. *A*: glycogen levels were measured in hearts from non-TG, WT-TG, and RG-TG mice. Results are means ± SE for 4 hearts and are plotted as micrograms of glycogen per milligrams of wet weight heart tissue. $*P < 0.05$ relative to non-TG mice; $***P < 0.005$ relative to non-TG or WT-TG mice. Transverse sections were taken and stained with periodic acid Schiff's (*B–D*) or hematoxylin and eosin (*E–G*). In both cases, ×400 magnification was used.

function or conduction abnormalities, although they did have moderately increased heart weight and glycogen accumulation compared with their non-TG littermates. These findings demonstrate that development of the disease phenotype is dependent on expression of mutant γ2. The RG-TG mice, therefore, provide a model system for investigating the mechanisms underlying the human disease.

There was a substantial increase in spontaneous mortality in RG-TG mice. Before their death, there were no obvious indicators of ill health. It is not known whether the sudden deaths indicate that the cause was cardiac arrhythmia or whether death occurred from progressive heart failure; deaths from both mechanisms have been noted in human subjects with γ2 mutations (3). Mortality was significantly higher in male mice versus female mice, although the increases in heart weight were similar in males and females [heart weight as %body wt at 8 wk: males = 1.3 ± 0.06 and females = 1.4 ± 0.09 (n = 5); at 16 wk: males = 1.4 ± 0.07 and females = 1.3 ± 0.13 (n = 4)]. It is possible that female sex hormones afford a cardioprotective effect, but we have not pursued this hypothesis.

The level of glycogen in hearts from RG-TG mice reached values in excess of 30 μg/mg wet weight, compared with 1–2 μg/mg in WT-TG hearts and ~0.4 μg/mg in non-TG hearts. It is unlikely that the increased glycogen content can account for the total increase in heart weight in the RG-TG mice. Heart weight increased approximately threefold in RG-TG mice compared with non-TG mice, without a detectable change in body weight. For a mouse with a body weight of 30 g, this represents an increase in total heart weight of 300 mg, far more than can be accounted for by glycogen. The most likely explanation is that in addition to glycogen, there is an increase

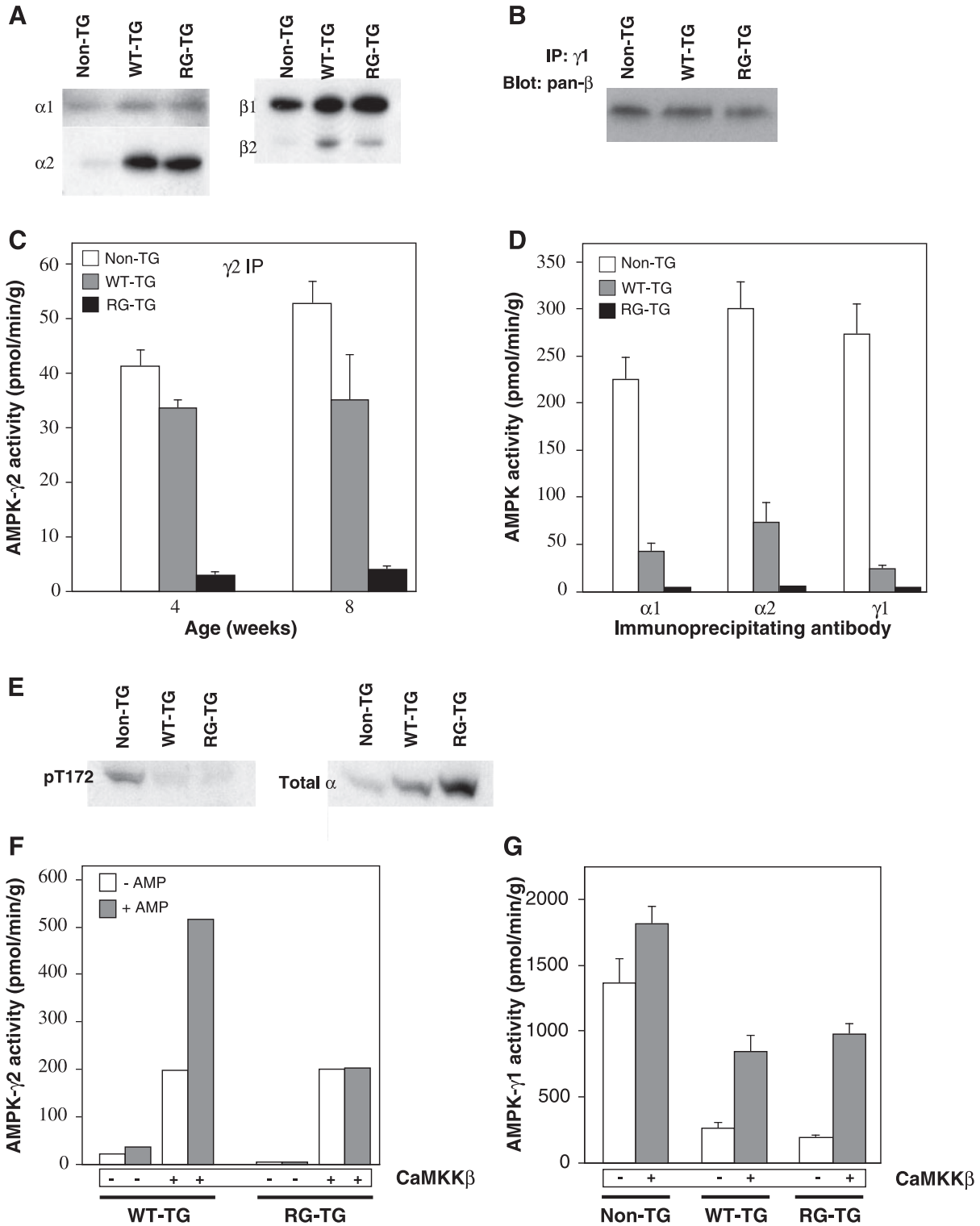

Fig. 5. AMP-activated protein kinase (AMPK) subunit expression and activity in heart. *A*: Western blot analysis of heart extracts (50 μg protein) from 8-wk-old animals probed with subunit-specific AMPK antibodies. Blots were visualized by enhanced chemiluminescence. *B*: AMPKγ1-containing complexes isolated by immunoprecipitation (IP), resolved on SDS-PAGE, and probed with a pan-β antibody. In each case, a representative blot is shown. *C*: AMPK activity in γ2-immune complexes isolated from heart extracts. *D*: AMPK activity in heart extracts (8-wk-old animals) in α1-, α2-, or γ1-immune complexes. *E*: Western blot analysis of heart extracts (50 μg protein) from 20-wk-old mice that were probed with either pan-α or phospho-T172 antibodies. Blots were visualized by enhanced chemiluminescence. *F* and *G*: AMPK present in immune complexes was incubated with MgATP in the presence or absence of recombinant Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK-β; 50 μg/ml). After immune complexes were extensively washed, AMPK activity in the immune complex was measured using SAMS peptide assay. *F*: AMPK isolated with γ2-specific antibodies. *G*: AMPK isolated with γ1-specific antibodies. Unless indicated otherwise, AMPK assays were carried out in the presence of 0.2 mM AMP.

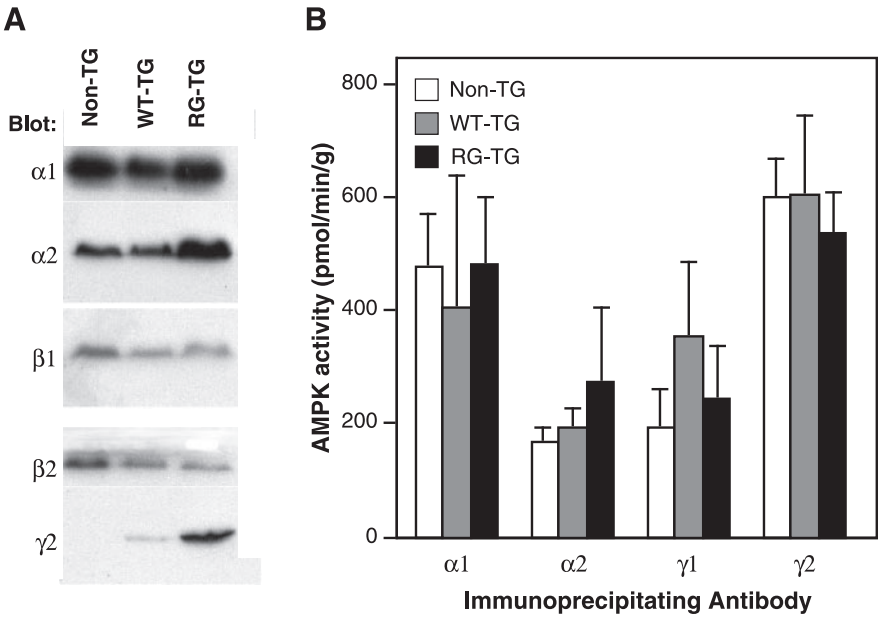

Fig. 6. AMPK subunit expression and activity in hearts from 1-wk-old mice. *A*: Western blot analysis of heart extracts (50 μg protein) from 1-wk-old mice probed with subunit-specific AMPK antibodies. *B*: AMPK activity in heart extracts from 1-wk-old mice measured in α1-, α2-, γ1-, or γ2-immune complexes in the presence of 0.2 mM AMP.

in protein mass within the heart. Consistent with this hypothesis, we observed increased expression of the hypertrophy-related genes, brain natriuretic peptide (BNP), and β-MHC in RG-TG hearts (BNP, 3.8- and 2.5-fold; and β-MHC, 16- and 13-fold increase relative to non-TG and WT-TG, respectively).

Analysis of the glycogen from RG-TG hearts revealed that it was less branched than the glycogen present in hearts of either non-TG or WT-TG mice. There are a number of glycogen storage diseases that are associated with decreased branching, including Andersen's disease (type IV) and Lafora's disease (10). Decreased branching in the glycogen from RG-TG hearts may have important implications in the pathology of the disease arising from γ2 mutations. Less branching decreases the solubility of the glucose polymers and reduces the number of nonreducing ends, decreasing the rate at which the polymers can be broken down and metabolized. One potential consequence of the decreased branching resulting from the γ2 mutation is that the polyglucosan accumulated in the heart will be more resistant than normal glycogen to degradation.

Two previous studies (2, 31) have reported on the generation of transgenic mice with cardiac-specific expression of AMPK-γ2 harboring different mutations from the one used in our current study. In both cases, expression of mutant γ2 resulted in a cardiac phenotype similar to the one we report here. In our current study, we were unable to detect any evidence of a cardiac phenotype at 1 wk; however, by 4 wk, RG-TG mice had developed a robust cardiac phenotype (hypertrophy, impaired cardiac function, and glycogen accumulation). Mice expressing the N488I mutation develop hypertrophy at ~8 wk and have impaired contractile function by 20 wk (2), whereas mice expressing the R302Q mutation begin to develop abnormal cardiac electrophysiology after ~3 wk (31). In the two previous studies, as well as our own, transgene expression was under the control of the α-MHC promoter. This promoter is switched on after birth, with barely detectable activity at 1 day but progressively increasing in activity, reaching a peak after 75 days, when activity remains high for up to 10 mo (28). This may explain, in part, the delayed onset of the phenotypes observed in the different transgenic models. To our knowledge, the regulation of γ2 expression during development in humans has not been reported. There is evidence, however, that indicates that the expression of endogenous γ2 is switched on relatively early in human development, because one individual with a H383R mutation was diagnosed clinically at birth (3). There is also evidence that, in humans, the nature of the mutation plays a role in the onset and progression of the disease. Individuals carrying the H383R or R531G mutations develop symptoms of WPW syndrome and cardiomyopathy in early childhood (3, 12), whereas individuals carrying the R302Q or N488I mutations typically present with symptoms in adolescence (11, 26). Although we are unable to determine directly whether the different transgenic γ2 models show a similar pattern in onset, it is interesting to note that the RG-TG mice generated in our study had a much earlier onset of mortality than was reported for the N488I transgenic mice (2).

There was a substantial increase in the protein levels of the α2-, β1-, and β2-AMPK subunits in both WT-TG and RG-TG hearts. This presumably occurs as a compensatory mechanism in response to the increase in γ2 expression in the transgenic animals, although we do not know the molecular basis underlying this effect. Expression of the γ2 transgene is under the control of the α-MHC promoter and is therefore likely to be restricted to cardiac myocytes. However, we cannot rule out the possibility that AMPK expression in other cell types, e.g., endothelial cells and smooth muscle cells, may also change in the transgenic models, confounding the interpretation. Interestingly, nonetheless, there was no decrease in γ1 expression, indicating that γ2 does not displace endogenous γ1 from existing AMPK complexes. A similar situation has been reported for transgenic mice expressing a dominant-negative form of AMPK-α2 in hearts. In that study (35), overexpression of dominant-negative α2 had no effect on the level of endogenous α1. These findings indicate that there must be some degree of selectivity in the formation of AMPK complexes in vivo. It is possible that increased AMPK subunit expression

may account for the mild phenotype observed in the WT-TG mice (increased heart weight and glycogen accumulation). WT-TG mice do not go on to develop the severe phenotype observed in RG-TG mice, providing unambiguous evidence that the mutation in γ2 is the causative effect leading to the disease.

The effect of disease-causing mutations in γ2 on AMPK activity remains controversial. It was reported that the N488I mutation led to an increase in AMPK activity (2), whereas the R302Q mutation was found to reduce activity (37). In view of these conflicting results, we carried out an extensive characterization of cardiac AMPK in the transgenic models in an attempt to better understand the effect of γ2 mutations on AMPK. A key finding from our study is that the activity of AMPK in hearts from transgenic mice varies with age. At 1 wk of age, there was no significant difference in AMPK activity in hearts from non-TG, WT-TG, or RG-TG mice at this stage. By 4 wk of age, however, AMPK activity in hearts from RG-TG mice was almost completely abolished and activity in WT-TG hearts was significantly reduced relative to non-TG levels. Our results strongly suggest that this reduction is not due to an intrinsic effect of the mutation in γ2 on AMPK activity per se, because the activity of γ1-containing AMPK complexes was reduced to the same level as γ2-containing complexes. The reduction in activity correlated with decreased phosphorylation of T172, and AMPK activity could be restored by phosphorylation with CaMKK-β, an AMPK-activating kinase (17, 19, 34). Consistent with previous studies (8, 30), we found that R531G γ2-complexes were insensitive to AMP stimulation.

A potential mechanism for the reduction in AMPK activity is that accumulation of glycogen, or a related metabolite, suppresses AMPK activation. Such a mechanism could account for a number of findings that we observe in our transgenic animal model. First, it could account for the delayed effect on AMPK activity, because glycogen levels do not begin to accumulate in the transgenic mice until 3–4 wk after birth. It could also account for our finding that total AMPK activity (γ1- and γ2-complexes) is reduced. Finally, the partial reduction in AMPK activity in WT-TG hearts could also be explained by this mechanism because cardiac glycogen in WT-TG mice reaches an intermediate level between RG-TG and non-TG mice. In skeletal muscle, AMPK activity was shown to be inversely correlated with the level of glycogen, suggesting that glycogen may directly affect the activation state of the kinase (32). Furthermore, AMPK activity in skeletal muscle of Hampshire pigs carrying the γ3 mutation, which causes increased glycogen accumulation, was reported to be significantly lower than in wild-type (24) mice. The mechanism by which glycogen, or a related metabolite, could act to suppress AMPK activation is unclear. Interestingly, however, the β-subunit isoforms contain a domain that is found in a number of enzymes that metabolize the α1–6 branch points in α1–4-linked glucans, which has recently been termed a glycogen-binding domain (18, 27), and it is possible that this domain could play some role in regulating AMPK activity in response to glycogen or glycogen-like polymers.

How do mutations in γ2 lead to increased glycogen accumulation? In contrast to a previous study (2) that reported increased AMPK activity in 1-wk-old hearts expressing γ2 N488I, we were unable to detect any change in AMPK activity in hearts from RG-TG mice compared with WT-TG or non-TG mice. It is possible that the R531G mutation results in a subtle change in activity at 1 wk of age that is not detected by the in vitro AMPK assay. Alternatively, it is conceivable that different mutations in γ2 could have distinct effects on AMPK activity and still result in virtually identical phenotypes. Perhaps more likely is that the disease-causing mutations in γ2 lead to accumulation of glycogen via a mechanism that is independent of their direct effect on AMPK activity. In this respect, it is interesting to note that a recent study (36) has reported that the pathogenesis of the phenotype resulting from mutation of N488I in γ2 may not be attributable to a simple loss or gain of function.

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