Constitutively active AMP kinase mutations cause glycogen storage disease mimicking hypertrophic cardiomyopathy

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Mutations in *PRKAG2*, the gene for the γ2 regulatory subunit of AMP-activated protein kinase, cause cardiac hypertrophy and electrophysiologic abnormalities, particularly preexcitation (Wolff-Parkinson-White syndrome) and atrioventricular conduction block. To understand the mechanisms by which *PRKAG2* defects cause disease, we defined novel mutations, characterized the associated cardiac histopathology, and studied the consequences of introducing these mutations into the yeast homologue of *PRKAG2*, Snf4. Although the cardiac pathology caused by *PRKAG2* mutations Arg302Gln, Thr400Asn, and Asn488Ile include myocyte enlargement and minimal interstitial fibrosis, these mutations were not associated with myocyte and myofibrillar disarray, the pathognomonic features of hypertrophic cardiomyopathy caused by sarcomere protein mutations. Instead *PRKAG2* mutations caused pronounced vacuole formation within myocytes. Several lines of evidence indicated these vacuoles were filled with glycogen-associated granules. Analyses of the effects of human *PRKAG2* mutations on Snf1/Snf4 kinase function demonstrated constitutive activity, which could foster glycogen accumulation. Taken together, our data indicate that *PRKAG2* mutations do not cause hypertrophic cardiomyopathy but rather lead to a novel myocardial metabolic storage disease, in which hypertrophy, ventricular pre-excitation and conduction system defects coexist.

Introduction

Dominant mutations in the γ2 regulatory subunit (*PRKAG2*) of AMP-activated protein kinase, an enzyme that modulates glucose uptake and glycolysis (1, 2), have been recently reported (3, 4) to cause hypertrophic cardiomyopathy (HCM). In addition to cardiac hypertrophy, individuals with *PRKAG2* mutations frequently manifest electrophysiologic abnormalities, particularly preexcitation (Wolff-Parkinson-White syndrome; WPW), atrial fibrillation, and progressive development of atrioventricular conduction block (3, 4). Although atrial fibrillation is common in HCM patients and becomes increasingly prevalent with disease duration, neither accessory pathways nor conduction system disease are typical features of HCM.

An autosomal dominant disorder, HCM is clinically recognized by unexplained ventricular hypertrophy and a distinctive histopathology that includes myocyte enlargement, myocyte disarray, and increased interstitial fibrosis (reviewed in ref. 5). Previous studies have identified ten different disease genes that are mutated in HCM; despite this genetic heterogeneity, a unified mechanism for disease has been postulated because each of the disease genes encodes a sarcomere protein (reviewed in ref. 6). The discovery of *PRKAG2* mutations appears to challenge the hypothesis that HCM is a disease of the sarcomere.

To understand the mechanisms by which *PRKAG2* defects cause disease we defined additional, novel mutations and examined the clinical manifestations found in affected individuals. A previously unrecognized and unusual histopathology was identified in hearts with *PRKAG2* defects, which prompted biochemical analyses of the functional consequences of human mutations on Snf4, the yeast homologue of the γ2 protein kinase subunit. Collectively our data indicate that *PRKAG2* defects do not cause HCM, but rather a novel glycogen storage disease of the heart.

Methods

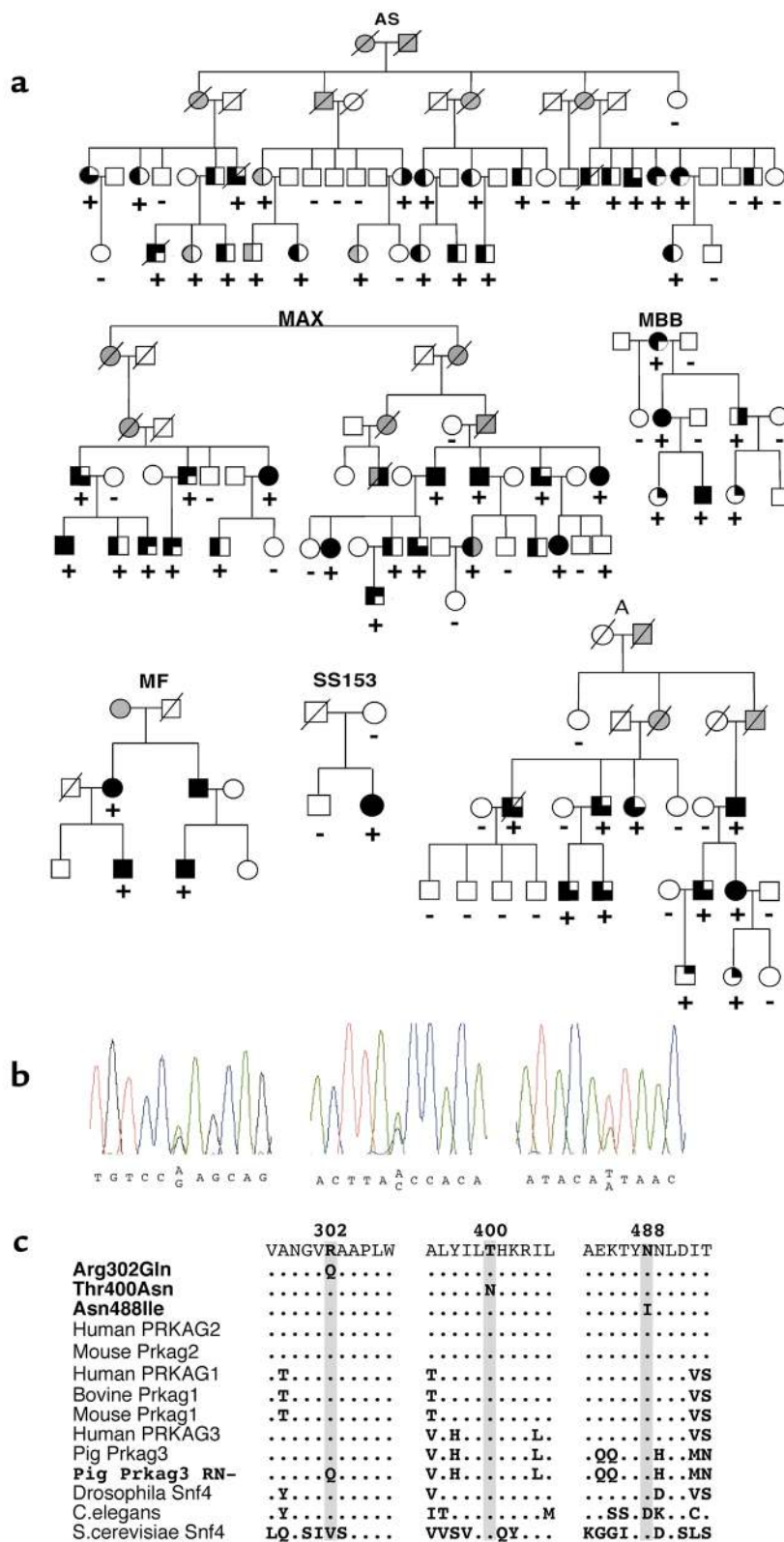*Clinical studies.* All studies were carried

Figure 1

Identification of three *PRKAG2* mutations inherited in six families and the clinical consequences of these mutations. (**a**) Pedigrees indicate clinical findings of cardiac hypertrophy (left half filled), WPW (right upper quadrant filled), or conduction system disease (right lower quadrant filled) in individuals with a *PRKAG2* mutation (+). Open symbols denote unaffected individuals, and shading denotes uncertain clinical status. (**b**) Sequence traces demonstrating G995A substitution (exon 7), C1289A (exon 11), and A1553T (exon 14), encoding Arg302Gln, Thr400Asn, and Asn488Ile substitutions, respectively. (**c**) Comparison of PRKAG protein sequences demonstrates evolutionary conservation of residues altered by mutation. Note that human *PRKAG2* Arg302Gln mutation is homologous to R200Q mutation in *RN⁻* pigs.

neous ventricular preexcitation, positive adenosine test, or demonstration of an accessory pathway on electrophysiologic study. Pathological specimens from members of families A (explanted heart), MF and SS (endomyocardial biopsies), and AS (necropsy specimens from two affected individuals) were examined using hematoxylin and eosin (H&E), Masson Trichrome, periodic acid Schiff (PAS), and PAS plus diastase stains. Samples for electron microscopic examinations were obtained from the paraffin-embedded tissue. After paraffin removal, tissue was fixed in 2% glutaraldehyde and postfixed in osmium tetroxide. Thin sections were stained with uracil acetate and lead citrate.

Genetic studies. Genomic DNA was extracted from whole blood or Epstein-Barr virus–transformed lymphocytes, as previously described (10). DNA from a deceased individual (Family A) was extracted from the pathological specimen. PRKAG2 cDNA sequence was blasted against the genomic sequence of the three bacterial artificial chromosomes (GenBank accession numbers: CAB65116; and AC006966, AC006358.5, AC074257, respectively) containing exons 1 and 4–16 to generate intronic primers. Genomic sequence encoding exons 2 and 3 was obtained from the Celera database (Celera Genomics, Rockville, Maryland, USA). Exons were PCR-amplified and sequenced using an ABI Prism 377 or ABI Prism 3700 DNA Analyzer

out in accordance with the institutional guidelines for human research. Review of medical records, clinical evaluation, and electrocardiogram and echocardiogram studies were performed as described (7, 8). Detailed

clinical characteristics for families AS and MAX were previously reported (8, 9). Cardiac hypertrophy was diagnosed on the basis of left ventricular wall thickness ≥ 13 mm. WPW was diagnosed based on documented sponta-

(Applied Biosystems, Foster City, California, USA). Primers sequences are available at http://genetics.med.harvard.edu/~seidman. Mutations were confirmed and families genotyped by restriction enzyme digestion. Sequence variants C1289A and A1553T, encoding Thr400Asn and Asn488Ile substitutions, respectively (Figure 1b), introduce a Tru9I site. To confirm G995A encoding Arg302Gln, a BsrBI restriction site was introduced into exon 7 PCR product using the reverse primer 5′-CTACAAAACTTTGTTTTTTACTCTCC-CACAGTGGCGCCGCT-3′. G995A abolishes this BsrBI restriction site. Haplotypes flanking G995A were characterized using single-nucleotide polymorphisms (SNPs) in introns 5 and 6, identified from the Celera database (Celera Genomics) using multiplex PCR (cv2667942-4, cv2667950-2, cv1222232-4) and sequence analyses. Intragenic tandem repeats (poly–CA/GT) were identified from the *PRKAG2* genomic sequence. Haplotypes were determined from PCR-amplified tandem repeats using fluorescent-labeled primers.

Yeast studies. Growth media were prepared as described (11). Plasmids pPS48 (encoding Snf4) and pGBT9-Snf1 and pPS50 (encoding GAD-Snf4) were generously provided by K.M. Arndt (12). Human mutations Thr400Asn and Asn488Ile were introduced into homologous locations 166 and 251 in *Snf4* by standard mutagenesis. *Saccharomyces cerevisiae* strain FY454a *his4-912δ, lys2-128δ, ura3-52, leu2Δ1, snf4Δ1* were transformed with pPS48 (wild-type), pPS48Thr400Asn, pPS48Asn488Ile, or pRS416 (snf4-deficient), grown on SC-Ura selection media. Colonies were then replica-plated on yeast extract peptone dextrose medium (YPD) with 2% glucose, glycerol, or raffinose as a carbon source. Raffinose plates contained 1 μg/ml antimycin A to inhibit mitochondrial phosphorylation. For two-hybrid studies, plasmids (12) (pGBT9-Snf1 + pPS50; pGBT9-Snf1 + pPS50Thr400Asn; pGBT9-Snf1 + pPS50Asn488Ile) and controls (pGBT9 + pGAD10; pGBT9-Snf1 + pGAD10; pGBT9 + pPS50; pGBT9 + pPS50Thr400Asn; pGBT9 + pPS50Asn488Ile) were cotransformed into *S. cerevisiae* Y190 (CLONTECH Laboratories Inc., Palo Alto, California, USA) and selected on synthetic dropout (SD) media depleted of Trp and Leu (SD-Trp-Leu). For quantitative assay, cells were grown to a mid-log phase in SD-Trp-Leu liquid media containing either Glu2% or galactose 2%/raffinose 1%. β-Galactosidase assays using chlorophenol-red-β-D-galactopyranoside as substrate and calculations were performed according to the manufacturer's instructions (CLONTECH Laboratories Inc.). β-Galactosidase activity did not exceed 0.5 units in any controls (data not shown).

Statistics. Two-point lod scores were calculated using an MLINK computer program assuming allele frequency 0.1% and 95% disease penetrance. The χ-square and Student's *t* tests were used for statistical comparisons.

Results

The *PRKAG2* gene from 18 probands with cardiac hypertrophy and WPW, or isolated, familial WPW, was screened for mutations by nucleotide sequence analyses. Sequence variants were found in six DNA samples from probands with both cardiac hypertrophy and WPW (Figure 1); four sequence variants occurred in families (A, AS, MAX, MBB) with disease linked to the *PRKAG* locus (8, 9). No muta-
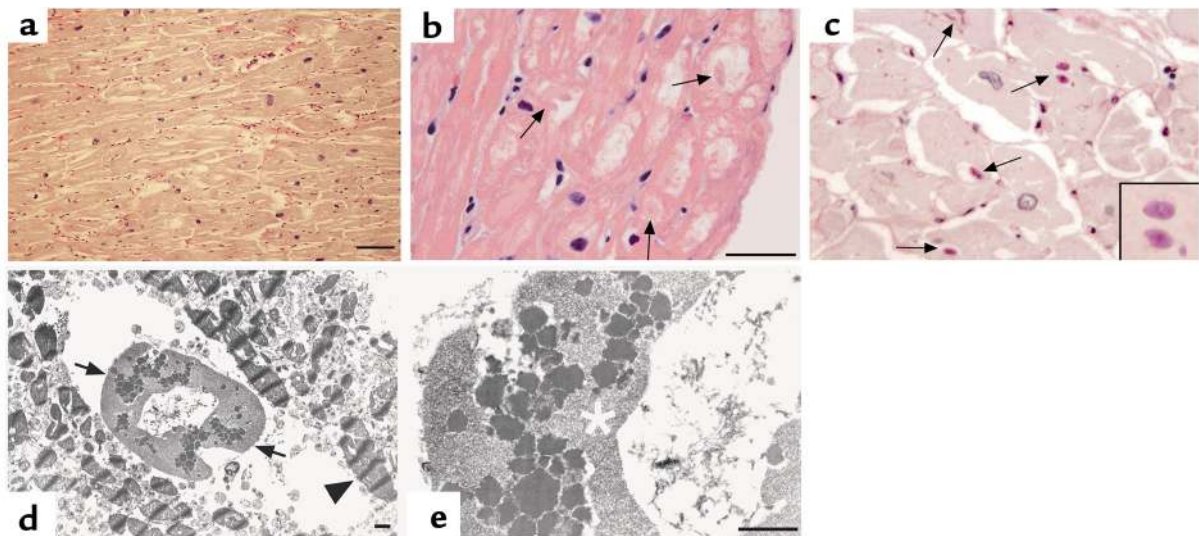
Figure 2
Histopathology of left ventricular sections obtained from individuals with *PRKAG2* mutations. (**a**) Longitudinal section of left ventricular myocardium from a 26-year-old individual with *PRKAG2* mutation Asn488Ile who died suddenly. Note vacuolated myocytes, and lack of myofiber disarray or fibrosis (H&E; bar = 100 μm). (**b**) High-power magnification of an endomyocardial biopsy (H&E; bar = 100 μm) from a 39-year-old individual with *PRKAG2* mutation Thr400Asn shows profound vacuolization (arrows). (**c**) Homogenous inclusions within vacuoles (arrows and inset) stained positive with PAS are mostly diastase-resistant. (**d**) Electron micrograph (uranyl acetate and lead citrate) of a sample described in **a** (bar = 1 μm). Note the large, irregular sarcoplasmic inclusion (arrows) within a large vacuole, and normal-appearing sarcomeres (arrowhead). (**e**) Higher magnification (bar = 1 μm) demonstrates that the inclusion is composed of a central core of homogenous, electron-dense droplets surrounded by osmiophilic granular and fibrillar material (star).

Table 1
Clinical features of individuals with *PRKAG2* mutations

Mutation	Arg302Gly	Thr400Asn	Asn488Ile	None
Family	A, MAX, MBB, MF	SS	AS	*n* = 12
Number affected	43	1	26	25
Age (years)	39 ± 16	42	32 ± 13	33 ± 18
LVH	74% (28)	(1)	76% (19)	36% (9)
LVWT (mm)	17.1 ± 7.6	22	19.3 ± 9.3	19.2 ± 13.3
Preexcitation	65% (28)	(1)	23% (6)[A]	88% (22)
Sinus bradycardia	12% (5)	(1)	31% (8)	(0)
AVB	37% (16)	(0)	8% (2)[B]	(0)
PPM	47% (20)	(1)	15% (4)[C]	(0)[D]
SCD	2	0	2	1

LVH, percentage (and number) of affected individuals with left ventricular hypertrophy (LVWT ≥ 13 mm); LVWT, left ventricular wall thickness; AVB, atrio-ventricular block; PPM, permanent pacemakers implanted; SCD, sudden cardiac death. Significant differences between electrophysiologic findings in individuals with Arg302Gln versus Asn488Ile mutations: [A]*P* = 0.002; [B]*P* = 0.01; [C]*P* = 0.02; and between individuals with versus without *PRKAG2* mutations: [D]*P* = 0.001.

tions were identified in 12 samples (Table 1) including three from probands of families with isolated WPW. Retrospective linkage analyses excluded the *PRKAG2* locus on chromosome 7q3 as the cause of disease in four kindreds, including two with isolated familial WPW (data not shown). Furthermore, *PRKAG2* mutations were not found in 20 unrelated probands with HCM unaccompanied by WPW or in 36 probands with familial dilated cardiomyopathy with or without conduction system disease.

All *PRKAG2* sequence variants encoded missense mutations. The previously reported (4) Arg302Gln substitution was found in four samples; analyses of flanking SNPs indicated that this defect arose independently in at least three of four samples (data not shown). Two novel mutations, Thr400Asn and Asn488Ile, were also identified (Figure 1b). All missense mutations were confirmed by restriction enzyme digestion and were absent

in more than 200 control samples. Missense mutations Arg302Gln and Asn488Ile cosegregated with affection status in study families (Figure 1a; lod scores = 12.1 [MAX, A, MBB, MF] and 8.4 [AS]; θ = 0). Thr400Asn was found in a proband with no other affected family members and may be a de novo mutation. Each of these three missense mutations altered highly conserved amino acid residues encoded in *PRKAG2* (Figure 1c).

The clinical consequences of three different *PRKAG2* mutations included cardiac hypertrophy, ventricular preexcitation (WPW), and progressive dysfunction of the conduction system. The presence of conduction system disease was the most significant clinical parameter delineating families with *PRKAG2* mutations among kindreds with WPW with or without hypertrophy (Table 1). WPW usually manifested early in life with tachyarrhythmias (atrial fibrillation and other supraventricular arrhythmias) and sometimes

caused syncope. Electrophysiologic studies in ten affected individuals demonstrated one or more accessory atrioventricular pathways. With increasing age, affected individuals progressively developed slower heart rates. Sinus bradycardia and/or variable degrees of atrioventricular block resulted in pacemaker implantation in 24 (35%) affected individuals. Left ventricular function markedly deteriorated in five affected adult individuals and necessitated cardiac transplantation in one; sudden death occurred in four. Few differences were observed between the clinical manifestations of the Asn488Ile and Arg302Gln mutations. Conduction disturbances may be more prevalent and severe in individuals with the Arg302Gln mutation, as reflected by more WPW and implantation of more permanent pacemakers, than in individuals with the Asn488Ile mutation.

To evaluate the effects of *PRKAG2* mutations on myocyte morphology,

Table 2
Effects of human *PRKAG2* mutations in Snf4 on yeast growth

Carbon source	Temperature (°C)	Snf4 variants			
		Δ	Wild-type	Thr400Asn	Asn488Ile
YPD + glucose	30	+++	+++	+++	+++
	39	++	+++	+++	+++
YPD + glycerol	30	±	++	++	++
	39	–	+	+	+
YPD + raffinose	30	–	++	++	++
	39	–	±	±	±

Growth of *S. cerevisiae* expressing wild-type or mutationally altered Snf4 on different carbon sources (see Methods) was assessed after 48 hours. –, Absent; ±, very weak; +, weak; ++, moderate; +++, good.

histopathology was examined in five cardiac specimens from affected individuals, ages 22–55. Comparable findings were observed in specimens with each of the three different *PRKAG2* missense mutations (Figure 2 and data not shown). Marked ventricular hypertrophy was evident on gross inspection, and myocytes were enlarged (Figure 2a), but myofiber disarray, the characteristic feature of HCM, was not detected in any sample. Interstitial fibrosis was minimal and focal. Surprisingly, all five specimens demonstrated isolated, large cytosolic vacuoles in cardiomyocytes (Figure 2b). These vacuoles contained inhomogeneous granular material that stained strongly with PAS (Figure 2c) and was diastase-resistant, a pattern that is characteristic of polyglucan (13). Electron microscopy of specimens revealed densely packed fine granular and fibrillar, electron-dense material within vacuoles (Figure 2, d and e), features that are characteristic of amylopectin, a nonsoluble product of glycogen metabolism. Such findings are typical of type IV glycogenosis and polyglucosan body disease and have also been found in hearts of patients with adult-onset Pompe disease (14–16).

Mammalian AMP-activated protein kinase is a heterotrimeric molecule with regulatory (γ) and enzymatic (α) subunits that are homologous to yeast Snf4 and Snf1 (1, 17), respectively. Two of three amino acid residues altered by human mutations (Asn488 and

Thr400; Figure 1c) are conserved in Snf4. To examine the effects of these disease-causing *PRKAG2* mutations on γ2 functions, we introduced Asn488Ile and Thr400Asn substitutions into Snf4 from *S. cerevisiae*. Yeast strains lacking Snf4 require glucose for growth (18). However, Snf4 engineered to contain either Thr400Asn or Asn488Ile allowed yeast growth comparable to that of strains containing wild-type Snf4 (Table 2), indicating that these mutant subunits were functional. Interactions between wild-type Snf4 and Snf1 are normally inhibited by glucose (12, 19), such that kinase activity is very low. However, in the presence of glucose, mutant Snf4Thr400Asn or Snf4Asn488Ile exhibited significantly stronger interaction with Snf1 than did wild-type Snf4 (Figure 3a). In glucose-deprived media, Snf1 interactions with Snf4 mutants was preserved or further increased (Figure 3b). We interpret these data to indicate that Thr400Asn and Asn488Ile mutations produce nonphysiologic, constitutive activation of AMP kinase.

Discussion

We demonstrate that missense mutations in the human γ2 regulatory subunit of AMP-activated protein kinase cause cardiac hypertrophy and electrophysiologic deficits, but not HCM. *PRKAG2* mutations produce a distinctive cardiac histopathology characterized by enlarged myocytes with vac-

uoles containing glycogen derivatives; notably, myocyte disarray is absent and interstitial fibrosis is minimal. Human *PRKAG2* defects introduced into the yeast homologue Snf4 result in constitutive enzyme activity. Collectively these data indicate that *PRKAG2* mutations cause a novel form of cardiomyopathy associated with polysaccharide storage in the heart.

Persistent activity of AMP kinase in the heart could account for dominant expression of a heterozygous mutation. Nonphysiologic activation of cardiac AMP kinase by these mutations should increase glucose uptake (by stimulating translocation of the glucose transporter GLUT-4 to the plasma membrane) and increase hexokinase activity, thereby leading to glycogen accumulation (20, 21). Polysaccharide storage and vacuole formation in myocytes containing a *PRKAG2* mutation (Figure 2) is therefore highly compatible with inappropriately increased baseline AMP kinase activity.

Support for the hypothesis that human *PRKAG2* missense mutations cause an increase in AMP kinase activity and stimulate carbohydrate accumulation also comes from the analyses of *RN⁻* pigs (22, 23). *RN⁻* pigs produce "acid meat" that is of inferior quality due to increased muscle glycogen content. Recent studies defined the *RN⁻* mutation to be a missense defect in γ3, a skeletal muscle AMP kinase subunit encoded by the *PRKAG3* gene. There is considerable homology between porcine
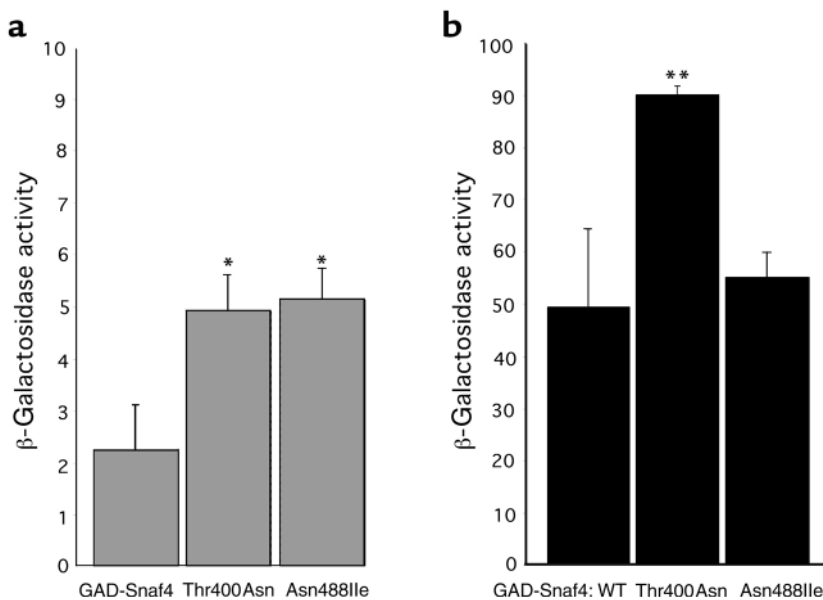
Figure 3
Snf1-Snf4 two-hybrid interactions in the presence and absence of glucose. β-Galactosidase activity was measured in cell cultures grown in SD glucose (**a**) or galactose/raffinose (**b**) media. The activity from three cultures from different colonies is reported in units (hydrolysis of 1 μmol CPRG/min/cell), mean ± SD. WT, wild-type. *$P < 0.02$, **$P < 0.005$ versus wild-type *Snf4*.

PRKAG3 and human *PRKAG2*, with 62% identity in a 316–amino acid region. The porcine *RN⁻* mutation is analogous to the human cardiac *PRKAG2* mutation Arg302Gln (Figure 1c).

Although human *PRKAG2* mutations have previously been reported to cause HCM and/or WPW (3, 4), our genetic, biochemical, and histopathologic data indicate that these defects actually produce a novel cardiac glycogen storage disease, manifested by cardiac hypertrophy and conduction system disease. Excess glycogen storage and water in cardiomyocytes appear to account for increased cardiac mass in affected individuals (24). Glycogen accumulation in conductive tissue is also likely to cause sinus and atrioventricular node dysfunction, electrophysiologic abnormalities that are atypical of HCM but are the distinguishing clinical features of *PRKAG2* mutations (Table 1). Electrophysiologic disturbances are frequent cardiac manifestations of other glycogen storage disorders such as Pompe disease and Danon disease (24–27). WPW has also been observed in several patients with glycogen storage disease (25, 26); however, neither electrophysiologic nor anatomical evidence of accessory pathways has been obtained in these patients (28). How glycogen accumulation accounts for the presence of atrioventricular accessory pathways associated with *PRKAG2* mutations remains an open question. Two mechanisms appear possible: either embryonic atrioventricular connections that normally regress during heart development persist in individuals with PRKAG2 mutations, or metabolic deposits activate quiescent accessory pathways. Although WPW that occurs without cardiac hypertrophy does not appear to be caused by *PRKAG2* mutations, understanding this mechanism may well provide insights into other etiologies of WPW.

We conclude that management strategies and prognostic information for individuals with *PRKAG2* mutations should not be predicated on HCM. We suggest that therapies directed toward reduction of AMP kinase activity may provide therapeutic benefit and alter the disease progression in these patients.

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