Functional Analysis of Mutations in the γ2 Subunit of AMP-activated Protein Kinase Associated with Cardiac Hypertrophy and Wolff-Parkinson-White Syndrome

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Mutations in the gene encoding the γ2 subunit of the AMP-activated protein kinase (AMPK) have recently been shown to cause cardiac hypertrophy and ventricular pre-excitation (Wolff-Parkinson-White syndrome). We have examined the effect of four of these mutations on AMPK activity. The mutant γ2 polypeptides are all able to form functional complexes following co-expression with either α1β1 or α2β1 in mammalian cells. None of the mutations caused any detectable change in the phosphorylation of threonine 172 within the α subunit of AMPK. Consequently, in the absence of an appropriate stimulus the mutant complexes, like the wild-type complex, exist in an inactive form demonstrating that the mutations do not lead to constitutive activation of the kinase. Three of the mutations we studied occur within the cystathionine β-synthase (CBS) domains of γ2. Two of these mutations lead to a marked decrease in AMP dependence, whereas the third reduces AMP sensitivity. These findings suggest that the CBS domains play an important role in AMP-binding within the complex. In contrast, a fourth mutation, which lies between adjacent CBS domains, has no significant effect on AMPK activity in vitro. These results indicate that mutations in γ2 have different effects on AMPK function, suggesting that they may lead to abnormal development of the heart through distinct mechanisms.

The AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade that plays a pivotal role in the regulation of energy metabolism (1). In response to activation following an increase in the AMP/ATP ratio, AMPK phosphorylates a number of downstream targets culminating in the switching off of energy (ATP)-utilizing pathways and the switching on of energy-generating pathways (1, 2). Activation of AMPK is complex and involves direct allosteric activation of the enzyme by AMP as well as phosphorylation, catalyzed by an upstream kinase, AMPK kinase (AMPKK) (3, 4). AMPK is a heterotrimERIC complex, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ) (5, 6) and isoforms of all three subunits have been identified (7–9). Although there is compelling evidence indicating that formation of the heterotrimeric complex is necessary for significant kinase activity (6, 10) the precise role of the regulatory subunits remains unclear. In addition, at present our understanding of the physiological relevance of the different subunit isoforms is very limited.

Several groups have reported recently the identification of six different mutations in the γ2 subunit from patients with cardiac hypertrophy and associated electrophysiologic abnormalities (11–14). All six mutations result in amino acid substitutions and are located within the C-terminal half of the protein. The mutations lead to the development of aberrant conduction systems, including pre-excitation, characteristic of Wolff-Parkinson-White syndrome (15) and in all but one case, the mutations also result in severe cardiac hypertrophy. No evidence of cardiac hypertrophy, however, was found in individuals carrying a mutation of arginine to glycine at residue 531 (R531G) (13). At present, the molecular mechanisms by which mutations in γ2 lead to abnormal conduction and hypertrophy are unknown. Interestingly, however, enlarged myocytes from an individual with a mutation in γ2 were found to have vacuoles containing glycogen derivatives, suggesting that the hypertrophy may be a result of increased carbohydrate storage (14). A mechanism involving increased glycogen storage is attractive, because an arginine to glutamine mutation at residue 226 (R226Q) in the γ2 isoform causes excess glycogen accumulation in pig skeletal muscle (16).

Three AMPK γ isoforms have been identified and all share a highly conserved C-terminal region of ~300 amino acids (9). Within this conserved region are four cystathionine β-synthase (CBS) domains (17). CBS domains have been identified in a wide range of proteins, but their function remains unknown. Mutations within the single CBS domain of human cystathionine β-synthase, from which the acronym stems, cause homocystinuria (18). Four of the mutations in γ2, together with the R226Q mutation in the γ2 isoform in pig, occur within the CBS domains. These findings raise the possibility that the CBS domains in AMPK may have an important functional role. It was reported that AMPK activity in skeletal muscle isolated from pigs harboring the R226Q mutation in γ2 was lower than that in the corresponding wild-type pigs (16). In that study, however, total AMPK activity, rather than γ2-specific activity was measured, making interpretation of the results difficult. In another study (19), the arginine residue in γ1 (Arg70) equivalent to Arg226 in γ2 was mutated to a glutamine residue in an effort to mimic the γ2 mutation. It was reported that this mutation caused a marked increase in AMPK activity and rendered it largely AMP independent (19).

The identification of mutations in γ2 that lead to severe heart

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† The abbreviations used are: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; SAM, the synthetic peptide HMRSAMSLHLVKRR; Thr172, threonine residue 172 within the α subunit of AMPK; CBS, cystathionine β-synthase.

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defects in humans has important implications for our understanding of the pathogenesis and treatment of cardiac hypertrophy and ventricular pre-excitation. The lack of information regarding the impact of these mutations on AMPK activity prompted us to carry out our current study. We report here the effect of four different mutations in \( \gamma_2 \), three that occur within the CBS domains and one that lies between adjacent domains, on the activity of AMPK in mammalian cells. None of the mutations in \( \gamma_2 \) that we studied cause a detectable increase in the phosphorylation of threonine 172 within the \( \alpha \) subunit of AMPK (Thr \(^{172} \)) compared with wild-type \( \gamma_2 \) complexes. Thus, in the absence of an appropriate stimulus to promote phosphorylation of Thr \(^{172} \), the mutant complexes, like the wild-type complex, exist in a relatively inactive form. These results demonstrate that the \( \gamma_2 \) mutations do not lead to constitutive activation of the kinase. Two of the three mutations occurring within the CBS domains cause a marked decrease in AMP activation of the kinase, whereas a third mutation in the CBS domain leads to a doubling of the \( A_{\text{ph}} \) for AMP. In contrast, the leucine insertion mutation that occurs between CBS domains 1 and 2 has no significant effect on AMP sensitivity in vitro. These results suggest that the pathways by which mutations in \( \gamma_2 \) lead to the pathogenesis of heart disease involve different mechanisms.

### EXPERIMENTAL PROCEDURES

#### Mutagenesis of \( \gamma_2 \) Subunit—Oligonucleotides spanning the sequence to be mutated, and including the appropriate base changes, were used to amplify cDNA encoding AMPK \( \gamma_2 \) (9) using a PCR-based strategy as described previously (20). A sequence encoding a FLAG epitope tag (amino acid sequence DYKDDDDK) was inserted immediately after the initiating methionine codon to facilitate detection of the \( \gamma_2 \) subunit. All cDNAs were cloned into pCDNA3 (Invitrogen) and sequenced completely on both strands to ensure their authenticity.

#### Mammalian Cell Expression—Plasmid DNA was prepared using a Qiagen maxi-prep kit according to the manufacturer’s instructions. CCL13 cells were co-transfected with cDNA (10 \( \mu \)g/plasmid) encoding either \( \alpha_1 \) or \( \alpha_2 \), and \( \beta_1 \) and \( \gamma_2 \) (all cDNAs cloned into pCDNA3) by calcium phosphate precipitation (21). The \( \alpha_1 \) and \( \alpha_2 \) cDNAs encode a Myc epitope tag (amino acid sequence EKQKLISEEDL) at the N terminus (20). Cells were harvested 72 h post-transfection by one of two methods. In the first method (rapid lysis), the culture medium was replaced with 1 ml of ice-cold lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10% (v/v) glycerol, 1% (v/v) Triton X-100) immediately added to the cells. Insoluble material was removed by centrifugation (13,000 \( \times g \), 1 min at 4 °C) and the supernatant fraction used for subsequent analysis. In some cases, cells were submitted to hyperosmotic stress, by addition of sorbitol (final concentration of 0.6 M) to the culture medium and incubated for 30 min immediately prior to lysis. In the second method, cells were allowed to become anoxic thereby leading to activation of AMPK in response to an increase in the AMP/ATP ratio. In this method, following removal of the culture media the cells were briefly rinsed with phosphate-buffered saline and then exposed to anoxic conditions for 2–3 h at 4 °C with 10 \( \mu \)l of a 50% (w/v) slurry of an antimycin A (clone 9E10 (22)). Cell lysates (0.1 ml for each complex) were incubated for 2 h at 4 °C with 10 \( \mu \)l of a 50% (w/v) slurry of antimycin A in 1 ml of lysis buffer and insoluble material was removed by centrifugation.

#### AMPK Activity—AMPK activity was determined in immunocomplexes isolated by immunoprecipitation from cell lysates using an anti-Myc monoclonal antibody (clone 9E10 (22)). Cell lysates (0.1 ml for each complex) were incubated for 30 min at 4 °C in the presence or absence of varying concentrations of AMP as described in the figure legends. Activities were calculated as picomole of phosphate incorporated into the SAMS peptide per minute per mg of total protein.

AMP dependence curves were generated using the equation \( v = b + [(s \times b) - b] \times [AMP] / [A_{\text{ph}} + [AMP]] \), where \( s \) is the relative stimulation, \( b \) is the basal activity, and \( A_{\text{ph}} \) is the concentration of AMP giving half-maximal stimulation, using Graphpad Prism software.

#### Western Blot Analysis—Proteins present in the immune complexes were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane. The membrane was blocked by incubation in 10 mM Tris-HCl, pH 7.4, 0.5% NaCl, 0.5% Tween 20, 5% low fat milk powder for 1 h at room temperature. Membranes were probed with an anti-Myc (9E10), anti-FLAG M2 (Sigma), or anti-AMPK P-Thr\(^{172} \) (Cell Signaling Technologies) antibody for 2–18 h at 4 °C in blocking buffer and then washed extensively with 10 mM Tris-HCl, pH 7.4, 0.5% NaCl, 0.5% Tween 20. The blots were incubated for 1 h at room temperature with either goat anti-mouse IgG (for anti-Myc and anti-FLAG blot) or donkey anti-rabbit IgG (AMPK\( \beta \) and AMPK phospho-Thr\(^{172} \)) (Cell Signaling Technologies) and chemiluminescence (Roche Molecular Biochemicals).

#### Yeast Complementation and Two-hybrid Analysis—A mutation in Snf4 cDNA was introduced by PCR to alter arginine residue 294 to glycine. snf4\( \Delta \) mutant yeast (strain MCV2634) were transformed with either wild-type or mutated Snf4 in pGAD424 (Clontech) and selected by growth on media lacking tryptophan. Transformants were patched onto solid media containing either 2% glucose or 2% raffinose as carbon sources. 

#### Statistical Analyses—Statistical significance was determined using a two-tailed unpaired Student’s \( t \) test.
FIG. 1. Location of mutations within AMPK γ2 used in this study. The location of the four mutations examined in this study are represented schematically in A. The four CBS domains within the C-terminal half of γ2 are shown as shaded boxes. In B the amino acid sequences of the CBS domains of human γ2 in which the R302Q (CBS1), H383R (CBS2), and R531G (CBS4) mutations are located, are shown aligned. The CBS domain of pig γ2 (CBS1), which contains the R226Q mutation, and the single CBS domain of human cystathionine β-synthase, which contains the D444N mutation, are also shown aligned. Amino acid identities in the CBS domains of the γ isoforms are shaded in black and conservative substitutions in gray. The residues at which the mutations occur are denoted by arrows above the sequences.

FIG. 2. Effect of γ2 mutations on AMPK activity in anoxic cells. Wild-type γ2 (WT) or mutant γ2 (R302Q, H383R, R531G, and L ins) were co-expressed with either α2β1 or α1β1 in CCL13 cells and AMPK complexes were immunoprecipitated from lysates prepared from anoxic cells. AMPK activity of α1-containing complexes (A) and α2-containing complexes (B) was determined in immune complexes using the SAMS peptide assay. Values shown are the mean ± S.E. from four independent experiments and are expressed as picomole/min/mg. Background activity present in immune complexes isolated from untransfected cells (UT) is also shown. Western blot analysis of proteins within the immune complexes is shown below the graphs. Blots were probed with anti-Myc (present on the α subunit), anti-phosphothreonine 172 (P-Thr172), anti-FLAG (present on the γ subunit), or anti-β subunit antibodies. In each case a representative blot is shown.
expected, virtually no AMPK activity or expression was detectable in immunoprecipitates isolated from untransfected cells.

In contrast to anoxic cells, AMPK isolated from rapidly lysed cells is relatively inactive because the intracellular ratio of AMP/ATP remains low (25). As can be seen in Fig. 3, the activity of α1-containing AMPK, measured in the presence of 200 μM AMP, isolated from rapidly lysed cells is very low in either wild-type or mutant γ2 complexes. Similar results were obtained for α2-containing complexes (results not shown). Consistent with the low activity of the complexes, no phosphorylation of Thr172 was detected in immunoprecipitates isolated from rapidly lysed cells, despite similar levels of expression of the complexes determined by Western blot analysis (Fig. 3). These results indicate that the γ2 mutations do not have any significant effect on the phosphorylation and activation of AMPK by AMPKK under basal conditions.

We next investigated the effect of the mutations on the allosteric activation of the kinase by AMP. Fig. 4, A and B, shows the activity of α1 (Fig. 4A) and α2 (Fig. 4B) complexes isolated from anoxic cells measured in vitro in the presence or absence of 200 μM AMPK. Qualitatively similar results were obtained for both isoforms, although the degree of stimulation by AMP was slightly greater for the α1 complexes compared with the α2 complexes. Based on these results, the activity of

**Fig. 3. Effect of γ2 mutations on basal AMPK activity and phosphorylation state.** Wild-type (WT) and mutant γ2 isoforms were co-expressed with α1, and AMPK activity in immune complexes isolated from lysates prepared from nonstressed CCL13 cells was measured in the presence of 200 μM AMP. Values shown are the mean ± S.E. from four independent experiments and are expressed as picomole/min/mg. Activities present in immune complexes isolated from either untransfected cells (UT) or anoxic cells expressing wild-type γ2 (WT anoxic, open bar) are shown for comparison. Proteins present in the immune complexes were subjected to Western blot analysis using either anti-Myc, anti-phosphothreonine 172 (P-Thr172) anti-FLAG, or anti-β subunit antibodies. In each case a representative blot is shown.

**Fig. 4. Effect of γ2 mutations on AMP activation of AMPK.** AMPK activity in immune complexes isolated from anoxic CCL13 cells expressing wild-type (WT) or mutant γ2 isoforms was measured in the presence (shaded bars) or absence (open bars) of 200 μM AMP. Results for α1-containing complexes (A) and α2-containing complexes (B) are shown. The values plotted are the mean ± S.E. from four independent experiments and are expressed as picomole/min/mg. C, AMPK activity of α1-containing complexes was determined over a range of AMP concentrations. Data were fitted to the equation: \( v = b + \frac{[s \times b - b] \times [AMP]}{A_{0.5} + [AMP]} \), where \( s \) is the relative stimulation, \( b \) is the basal activity, and \( A_{0.5} \) is the concentration of AMP giving half-maximal stimulation, using Graphpad Prism software. The data plotted are the mean ± S.E. from three independent experiments and are expressed relative to the activity in the absence of added AMP. The theoretical curves were fitted using the curve-fitting program. In all cases, similar amounts of AMPK were used in the assays, as judged by Western blot analysis of the immune complexes (data not shown).
the kinase measured in the presence, relative to the absence, of 200 \mu M AMP is shown in Table I. As can be seen, the R302Q, H383R, and R531G mutations all cause a significant reduction in AMP stimulation measured at 200 \mu M AMP. This is particularly evident with the R531G mutation, which almost completely abolishes activation by AMP. In contrast, the L ins mutation did not cause a significant difference in AMP stimulation measured at 200 \mu M AMP. It is noteworthy that following expression with \( \alpha_1 \), the activity of the R531G mutant is increased relative to the other complexes when measured in the absence of AMP (Fig. 4A). This is unlikely to be because of an increase in the phosphorylation state of Thr\(^{172} \) because we did not detect any change in phosphorylation of this residue between the different complexes (see Fig. 2A). One potential explanation for the increased activity is that the R531G mutation reduces the inhibitory effect of ATP on the kinase. Previous studies have shown that the allosteric activation of AMPK is antagonized by ATP (4) suggesting that both AMP and ATP bind to the same site. It is possible, therefore, that mutations that affect AMP-binding could also affect ATP binding. If this were the case for the R531G mutation it could account for the increased activity observed in the absence of AMP. We have not been able to address this possibility directly because of the fact that measurement of AMPK utilizes radiolabeled ATP in the assay, which raises a number of technical problems associated with varying ATP concentrations.

To investigate in more detail the effect of the mutations on AMP activation, \( \alpha_1 \) complexes were assayed over a range of AMP concentrations (Fig. 4C) and the concentration of AMP giving half-maximal stimulation (\( A_{0.5} \)) was calculated. For the wild-type complex an \( A_{0.5} \) of 77 ± 17 \mu M (\( n = 3 \)) was determined. The R302Q mutation (\( A_{0.5} \) 76 ± 14 \mu M; \( n = 3 \)) and L ins mutation (\( A_{0.5} \) 109 ± 20 \mu M; \( n = 3 \)) had no significant effect on this parameter. The H383R mutation, however, causes a large and statistically significant increase in the \( A_{0.5} \) value (177 ± 13 \mu M; \( n = 3 \), \( p < 0.05 \)) compared with the wild-type, accounting for the decrease in AMP stimulation observed at 200 \mu M AMP (Fig. 4A and Table I). At 500 \mu M AMP the activity of the H383R mutant is similar to that of the wild-type (Fig. 4C), indicating that although the AMP sensitivity is reduced, the AMP dependence is not significantly affected. The R531G mutation almost completely abolishes AMP stimulation and we were unable to determine accurately the \( A_{0.5} \) for AMP for this complex. These results strengthen the hypothesis that the CBS domains play an important role in the allosteric activation of the kinase by AMP, but do not reveal the mechanism by which the L ins mutation alters AMPK function.

We recently reported that AMPK is activated by two distinct pathways, one that involves changes in the AMP/ATP ratio and one that is independent of this ratio (26). Hyperosmotic stress activates AMPK, concomitant with an increase in the phosphorylation state of Thr\(^{172} \), without any detectable change in the AMP/ATP ratio (26). The finding that two of the \( \gamma_2 \) mutations have a marked effect on AMP activation is of particular interest. The AMPK complexes isolated from hyperosmotically stressed cells in the presence and absence of AMP were incubated in the presence (Sorbitol) or absence (Control) of 0.6 M sorbitol for 30 min prior to rapid lysis. AMPK complexes were isolated by immunoprecipitation and activity was determined in the presence or absence of 200 \mu M AMP, as indicated on the graph. Results shown are the mean ± S.E. from three independent experiments and are plotted as picomoles/min/mg. Western blot analysis of the complexes revealed similar levels of protein expression in each case (data not shown).

Fig. 5. Effect of hyperosmotic stress on AMPK activity of \( \gamma_2 \) mutant complexes. Wild-type (WT) and mutant \( \gamma_2 \) isoforms were co-expressed with \( \alpha_1 \beta_1 \) in CCL13 cells. Cells were incubated in the presence (Sorbitol) or absence (Control) of 0.6 M sorbitol for 30 min prior to rapid lysis. The activity of the R302Q and R531G mutants is increased relative to wild-type when assayed in the absence of AMP, whereas the activity of the H383R and L ins mutants is the same as wild-type. The increased activity of the R302Q and R531G mutants may be because of a reduction in AMP activity as described previously. In the presence of AMP, the activity of the R302Q, H383R, and R531G mutants is approximately half that of the wild-type and L ins complexes, similar to the situation observed in anoxic cells.

AMPK is structurally and functionally related to the SNF1 kinase in *Saccharomyces cerevisiae* (1). Yeast contain a single \( \alpha \) subunit (Snf1), a single \( \gamma \) subunit (Snf4), and three isoforms of the \( \beta \) subunit (Sip1, Sip2, and Gal83). Like the AMPK \( \gamma \) subunit isoforms, Snf4 contains four CBS domains. Alignment of the CBS domains in \( \gamma_2 \) and Snf4 revealed that arginine 531 in \( \gamma_2 \) is conserved in Snf4 (.argine 294), whereas the residues equivalent to arginine 302 and histidine 383 are not conserved (19). We mutated arginine 294 to glycine in Snf4 and transformed a yeast snf4 deletion strain with wild-type or mutant Snf4. Both wild-type Snf4 and Snf4R294G were able to rescue the growth of snf4 yeast on glycerol and raffinose media (Fig. 6A), indicating that the mutated Snf4 is capable of forming a functionally competent SNF1 complex in vivo. Snf4 interacts with Snf1, and this interaction is markedly increased in glucose-deprived cells (27). We examined the interaction of wild-type and mutant Snf4 with Snf1 in the two-hybrid system. The interaction of both wild-type and mutant Snf4 was low in cells grown in glucose and in both cases this interaction was in-
FIG. 6. Examination of an R294G substitution on Snf4 function in yeast. Arginine residue 294 in Snf4 (equivalent to Arg531 in \( \gamma_2 \)) was mutated to glycine. A, snf4 yeast were transformed with wild-type (WT) Snf4, mutant (R294G) Snf4, or empty vector (pGAD424), plated on selective media containing either 2% glucose, 5% glycerol or 2% raffinose as the sole carbon source and incubated for 5 days at 30 °C. B, yeast strain SFY526 was co-transformed with Snf1 (in pGBT9) and either wild-type (WT) Snf4 or mutant (R294G) Snf4 (in pGAD424). Interactions were determined by \( \beta \)-galactosidase liquid assays in permeabilized cells. Activities shown are the average values from duplicate assays that varied by less than 10% and are plotted as Miller units.

Discussion

In this study we have examined the effect of four mutations within the \( \gamma_2 \) subunit on the activity of AMPK. These mutations were first identified in individuals with abnormal cardiac function, including pre-excitation (Wolff-Parkinson-White syndrome) and hypertrophy (11–13). None of the mutations caused constitutive activation of AMPK as judged either by direct measurement of activity or by the phosphorylation state of Thr\(^{172} \). Rather, changes in Thr\(^{172} \) phosphorylation in response to either anoxia or hyperosmotic stress were remarkably similar in both the wild-type and mutant complexes. Our results differ from those of a previous study (19) where it was reported that mutation of arginine to glutamine at residue 70 (R70Q) within the \( \gamma_2 \) isoform, which is equivalent to arginine 302 in \( \gamma_2 \) and arginine 226 in \( \gamma_2 \), caused a marked activation of AMPK. In that study, phosphorylation of Thr\(^{172} \) within the \( \alpha_1 \) subunit of the R70Q-\( \gamma_1 \) mutant complex was significantly increased relative to the wild-type complex. The reason for the difference between our results and those of Hamilton et al. (19) remains unclear and further studies will be required to resolve these apparent discrepancies.

Whereas we did not find any difference in phosphorylation of AMPK, three of the mutations did have a significant effect on kinase activity in vitro. AMPK is routinely assayed in the presence or absence of 200 \( \mu \)M AMP and at this concentration the R302Q, H383R, and R531G mutations all caused a significant reduction in the degree of stimulation by AMP. These results are consistent with the effect of the R70Q mutation in \( \gamma_2 \), which reduced AMP activation by approximately half (19). Determination of the kinetic parameters involved in AMP activation demonstrated that the R302Q and R531G mutations cause a marked reduction in the AMP dependence of the kinase. The H383R mutation, however, increases the \( K_d \) for AMP, but has no significant effect on overall AMP dependence. At high concentrations of AMP (500 \( \mu \)M) the activity of the H383R complex is similar to wild-type. The concentrations of AMP required for half-maximal stimulation of the \( \gamma_2 \) complexes are significantly higher than those previously reported for \( \gamma_1 \)-containing AMPK complexes. Immunoprecipitation of \( \alpha_1 \) and \( \alpha_2 \) complexes from rat liver, in which \( \gamma_1 \) accounts for over 90% of the total AMPK activity (9), yielded \( K_d \) values for AMP of 12 ± 3 and 22 ± 3 \( \mu \)M, respectively (30). We reported similar values for \( \alpha_1 \) (5.7 ± 2 \( \mu \)M) and \( \alpha_2 \) (16 ± 3.5 \( \mu \)M) complexes following co-expression with \( \beta_2 \gamma_2 \) in mammalian cells (20). Our results indicate, therefore, that \( \gamma_2 \)-containing complexes are less sensitive to AMP than the corresponding \( \gamma_1 \) complexes. This finding may have important implications when considering the physiological role of the different AMPK complexes. The relatively high concentration of AMP required to activate \( \gamma_2 \) complexes would suggest that these complexes could respond to a greater range of AMP concentrations compared with \( \gamma_1 \). This could be important in tissues such as heart, where large fluctuations in adenosine nucleotides may occur. For instance, following 12 min of cardiac ischemia, AMP increased from around 1 to 143 \( \mu \)M, whereas ATP levels fell well below millimolar concentrations (31). Under these conditions, \( \gamma_1 \) complexes would be predicted to be maximally active, whereas \( \gamma_2 \) complexes would still be within a responsive range. Mutations in \( \gamma_2 \) that affect AMP activation of the kinase could have a significant effect on activity across this concentration range.

Our results provide further evidence for a role of the CBS domains in the allosteric regulation of AMPK by AMP. CBS domains occur in many proteins, and although the function of these domains is unknown it appears that they play a regulatory role, rather than a catalytic one. In human cystathionine \( \beta \)-synthase mutation of aspartic acid to asparagine at residue 444 (D444N) abolishes activation by S-adenosylmethionine, without affecting the basal catalytic activity (18). Intriguingly, this mutation lies in the equivalent position to the R302Q and H383R mutations within the CBS domain (see Fig. 1). These
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findings strongly suggest that residues at or near this position in the CBS domain have important functions in binding adenosine derivatives. Previously, we presented evidence that the γ2 subunit binds AMP (9), although we did not attempt to identify the residues involved in nucleotide binding. Because the γ isoforms each contain four copies of the CBS domains, it is possible that they bind more than one AMP molecule per subunit, and this could explain our finding that mutations within CBS1, -2, and -4 all have an effect on AMP activation. The varying effects on AMP activation produced by mutations in the different CBS domains may indicate that the four domains are not functionally equivalent in terms of AMP binding. Alternatively, it is possible that the differences may be because of the specific nature of the mutations. The R302Q and R531G mutations alter a basic arginine residue of an uncharged residue. It seems reasonable to predict that this could have a marked effect on ionic interactions, either within the AMPK complex or with AMP itself. On the other hand, substitution of histidine by arginine in the H383R mutation is a more conservative change and may have less of an effect on ionic interactions. Understanding the precise effect of these mutations at the molecular level will require detailed structural information from x-ray crystallographic studies.

An interesting observation that emerges from our study is that the phosphorylation of Thr172 appears to be unaffected by the γ2 mutations, despite the fact that some of these mutations bring about large changes in the AMP dependence of the kinase. Previous studies have shown that AMP makes AMPK a better substrate for phosphorylation by the upstream kinase in the cascade, AMPKK (30, 32). As a consequence of this it might be possible that the differences may be because of the specific nature of the mutations. The R302Q and R531G mutations would effect phosphorylation of Thr172, because they clearly reduce AMPK activity. That this is the case suggests that either the effect of AMP on AMPK activity and phosphorylation are distinct, or that direct activation of the upstream kinase by AMP (32) overrides the substrate-mediated effect of AMP on phosphorylation.

Recently, two further mutations in γ2 were identified in individuals with Wolf-Parkinson-White syndrome and associated cardiac hypertrophy (14). Based on results obtained with Snf4, the yeast equivalent of the γ subunit (1), it was reported that these mutations, T400N and N488I, caused constitutive activation of AMPK (14), although direct measurement of AMPK activity was not reported in this study. Introduction of these equivalent mutations into Snf4 increased the interaction of Snf4 with Snf1, the yeast equivalent of the α subunit, in yeast grown in the presence of glucose as determined by two-hybrid analysis. We introduced the equivalent of the R531G mutation into Snf4, but did not observe any change in the interaction of Snf1 and Snf4 in cells grown in the presence or absence of glucose. Therefore, if there is an effect of the equivalent γ2 mutations within Snf4 on its interaction with Snf1 it does not appear to be a common feature of all the mutations. The T400N mutation occurs toward the end of CBS domain 2, whereas the N488I mutation lies between CBS domains 3 and 4. Because these mutations do not align with any of the four mutations we examined it remains possible that they could have a different effect on AMPK activity and it will be important to determine directly the effect of the T400N and N488I mutations on AMPK activity.

In our current study we have been unable to detect a direct effect of the L ins mutation on AMPK activity. It is possible that this mutation has a subtle effect on activity that was not detected in the in vitro assays. Alternatively, this mutation may affect a function of AMPK that was not measured in our system, such as substrate binding. Clearly, further studies are required to investigate these possibilities. The remaining three mutations alter AMP activation of the kinase, such that under conditions that stimulate the kinase cascade via an increase in the AMP/ATP ratio, AMPK activity relative to wild-type. Conversely, in response to stimuli that promote phosphorylation of Thr172 without altering the AMP/ATP ratio, AMPK activation of the upstream kinase by AMP (32) overrides the substrate-mediated effect of AMP on AMPK activity. These results are somewhat paradoxical at first sight, but may be because of changes not only in AMP-binding but also in ATP-binding in these mutant complexes. The allosteric activation of AMPK is antagonized by ATP (4) and the simplest hypothesis is that AMP and ATP compete for the same allosteric site within the kinase. If this is the case it is likely that mutations that affect AMP binding will also affect ATP binding. Our results fit a model in which the basic residues (arginine or histidine) that are mutated within CBS domains 1, 2, and 4 of γ2 (see Fig. 1B) are involved in nucleotide binding. In this model, substitution of these residues reduces the affinity for both AMP and ATP, although the effects need not necessarily be equivalent for either the different mutations or the effect of the mutations on AMP and ATP. Based on this model, the effect of the individual mutations on AMPK activity would depend on the ratio of AMP/ATP within the cell, or in the in vitro assay. Although we have been able to study the effect of altering AMP within the assay we have not been able to determine the effect of changing ATP concentrations because of technical difficulties. Clearly, however, the important challenge is to determine the effect of the mutations on AMPK activity in vivo. The generation of animal models expressing the various γ2 mutations will facilitate a clearer understanding of these complex issues.

To our knowledge our results provide the first direct demonstration of the effects of naturally occurring mutations within γ2 on AMPK activity. These findings should facilitate further studies aimed at defining the molecular mechanisms by which mutations in γ2 lead to heart disease. A key question that arises from our study is how would a reduction in AMP dependence or AMP sensitivity of γ-containing complexes lead to the observed phenotype of pre-excitation and aberrant atrioventricular conduction, combined with cardiac hypertrophy? We have previously shown that in rat, γ accounts for only a small proportion (–10%) of total AMPK activity in most tissues, including heart (9). Although there have been no comparable studies in humans, given the close similarity between the rat and human AMPK complexes, it seems likely that this will also be the case in humans. Combined with the observation that mutations in γ2 are inherited in a dominant manner (11, 12), these findings indicate that a relatively small reduction in total AMPK activity within the cell can lead to a severe pathophysiological condition. Taken together with the knowledge that the γ2 mutations cause a cardiac-specific defect, and do not cause detectable abnormalities in other tissues, these characteristics indicate that γ-containing AMPK complexes must play a unique role in heart development, which cannot be compensated for by the other γ isoforms. AMPK has been implicated in the regulation of gene and protein expression (33–37) and it is possible that γ-containing complexes could play a role in the regulation of cardiac-specific proteins that are required for normal development of the heart conductance system. The γ2 polypeptide has an N-terminal extension of ~300 amino acids that is not present in either the γ1 or γ3 isoforms (9) and it is conceivable that this is involved in determining substrate recognition. Combined with phosphorylation of cardiac-specific proteins required for heart development, this could account for
the isofrom and tissue specificity of the \( \gamma_2 \) mutations. We are currently exploring these scenarios by generating animal models with genetically altered \( \gamma_2 \) subunits.

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