An activating mutation in the γ1 subunit of the AMP-activated protein kinase

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Abstract The AMP-activated protein kinase (AMPK) is a heterotrimeric protein composed of a catalytic α subunit and two regulatory subunits, β and γ. The γ subunit is essential for enzyme activity by virtue of its binding to the C-terminus of the α subunit and appears to play some role in the determination of AMP sensitivity. We demonstrate that a γ1R70Q mutation causes a marked increase in AMPK activity and renders it largely AMP-independent. This activation is associated with increased phosphorylation of the α subunit activation loop T172. These in vitro characteristics of AMPK are also reflected in increased intracellular phosphorylation of one of its major substrates, acetyl-CoA carboxylase. These data illustrate the importance of the γ1 subunit in the regulation of AMPK and its modulation by AMP.

Key words: Protein kinase; AMP; AMP-activated protein kinase; Cystathionine β-synthase; Cystathionine β-synthase domain

1. Introduction

The AMP-activated protein kinase (AMPK) is a heterotrimeric protein, composed of α, β and γ subunits, involved in regulating several metabolic pathways in response to cellular stress [1–4]. Under conditions of metabolic substrate limitation, hypoxia, exercise and heat-shock, AMPK is activated allosterically by rising cellular AMP and by phosphorylation of the catalytic α subunit by one or more AMPK kinases (AMPKKs) on Thr172, located in the 'T-loop' of the catalytic domain [1–8]. The α subunit has little activity as a monomer, but its activity increases dramatically following the binding of the non-catalytic β and γ subunits to its C-terminus [9–11]. The β and γ subunits also contribute to the stability of the α subunit [9]. At present two α (α1, α2), two β (β1, β2) and three γ (γ1, γ2, γ3) isoforms have been identified with tissue-specific heterogeneity of heterotrimer content and subcellular distribution [10,12–18]. In addition to regulation by phosphorylation of the α subunit, the β subunit is modified by post-translational myristoylation and by multi-site phosphorylation, which contribute both to enzyme activity and subcellular localization [19,20].

The γ subunit is the mammalian homologue of the Snf4 protein (Snf4p) of *Saccharomyces cerevisiae*, which is critical for the activity of the Snf1 protein kinase, a kinase central to metabolic adaptation in yeast [21,22]. The γ subunit binds both to the α and β subunits and appears to have some role in AMP sensitivity of the heterotrimer [9,11,16]. Heterotrimer reconstituted with different γ subunits is activated allosterically in vitro in a differential pattern, dependent on the presence of γ1, γ2, or γ3, the latter giving the least degree of AMP dependence [16]. The γ subunits are not post-translationally modified, save for an N-terminal acetylation noted in γ1 [19]. Sequence analysis of the γ subunits has identified that each isoform contains four tandem motifs termed cystathionine β-synthase (CBS) domains, initially identified in the enzyme, CBS [23]. CBS domains have been identified in other proteins as well, including voltage-gated chloride channels and inosine-5′-monophosphate dehydrogenase, but their function in any of these proteins remains unknown. Two different mutations within the CBS domains of human CBS gene (D444N and V453E) cause the disease homocystinuria [24]. Recently, a non-conservative substitution (R200Q) in the first CBS domain of the Hampshire pig PRKAG3 gene, the porcine homologue of γ3 subunit, was shown to result in a phenotype associated with high glycogen content in skeletal muscle [25].

We noted that the equivalent of the R200 residue in the first CBS domain of γ3 was preserved in all of the mammalian γ isoforms. Since the γ1 subunit is the most widely expressed γ subunit of AMPK (γ3 being largely restricted to skeletal muscle), we have elected to explore the impact of a CBS domain mutation in this protein. We have investigated this mutation by transient transfection of cDNAs, exploring its impact on AMPK heterotrimer assembly, enzyme activity, α subunit phosphorylation and AMP dependence of the isolated enzyme. We have also created cell lines stably expressing a γ1 mutant to observe its effect on cellular phenotype. These data indicate that γ1 R70Q mutation causes a dramatic activation of the αβγ heterotrimer, with increased phosphorylation of its α subunit and substantial loss of AMP dependence,

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Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-amino-4-imidazolecarboxamide-riboside; AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; CBS, cystathionine β-synthase; HA, hemagglutinin; NHE, Na^+/H^+ exchanger; PRKAG3, Hampshire pig AMPK γ3 isoform

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indicating an important role for the first CBS domain in the function of the AMPK γ subunit.

2. Materials and methods

2.1. Site-directed mutagenesis

Generation of the R70Q mutant was performed using a QuikChange™ site-directed mutagenesis kit (Stratagene) as described by the manufacturers. The complementary primers used to introduce the mutation were: 5′-GGTGACTAACGGTGTACAAGCTGCCC-CTTTATGGGATAG-3′ and 5′-CTATCCCATAAAGGGGCAGC-TTGTACACCGTTAGTCACC-3′, with the base change introduced underlined. The template for mutagenesis was the plasmid pMT2-HA-γ1 previously described containing the human γ1 isoform with a hemagglutinin (HA) tag generated at the 5′-terminus [5]. Following mutagenesis a *Bgl*II cassette (439 bp) from the confirmed R70Q mutant was used to replace the corresponding region in the original pMT2-HA-γ1 construct. The cassette region bearing the mutation was completely sequenced by *Taq* DyeDeoxy terminator cycle sequencing using an Applied Biosystems Model 373A Sequencer.

2.2. Transient expression and analysis of AMPK in COS cells

COS7 cells were cultured and transfected with cDNAs using FUGENE® reagent, as in [5]. The AMPK subunits were expressed in pEBG, pBK-CMV and pMT2 for the α1, β1 and γ1 subunits, respectively, as described previously [5]. Cell lysis, glutathione-agarose purification of expressed kinase heterotrimer, immunoblotting and measurement of AMPK activity have likewise been previously described [5]. Heterotrimer composition was assessed by immunoblotting of individual subunits with antibodies specific for each. The anti-α antibody anti-α(2–20) was generated by immunization with a peptide encompassing amino acids 2–20, common to both the α1 and α2 subunits. The anti-β antibody was raised against a bacterially expressed β1 protein. The anti-γ1 antibody was raised against a synthetic peptide encoding residues 319–331 of the human γ1 subunit, while an anti-HA antibody was used to probe for epitope-tagged γ1. The phosphorylation state on T172 of the α1 subunit was assessed by immunoblotting with an antibody specific for a phosphorylated T172 epitope.

2.3. Stable expression of AMPK γ1 subunits in pulmonary fibroblasts

For stable expression, PS120 cells lacking the Na^+/H^+ antiporter (Na^+/H^+ exchanger (NHE)) were transfected in 6-well plates, using LipofectAMINE® reagent [26]. Per well, we co-expressed 0.25 μg of pECE-NHE3 and either 1.75 μg of pMT2-HA-γ1 wild-type (WT) or HA-R70Q-γ1 or expressed 0.25 μg of pECE-NHE3 alone to create a control line. Two days after the transfection, the cells were exposed to the 'H^+-killing' selection test that eliminates cells that did not express a functional NHE [27]. Following two subsequent rounds of acid selection, lysates of pools of acid-selected cells were screened by immunoblotting with anti-HA antibody to identify the expression of the γ constructs. Having identified pools expressing the γ constructs, individual clonal lines were then isolated by limiting dilution. In the experiments reported herein, we used one isolate of control cells and two different clonal lines each of WT γ1 and R70Q mutant.

2.4. Characterization of stably expressing cell lines

The clonal lines of PS120 cells were characterized with respect to AMPK activity and heterotrimer composition, the phosphorylation state of the α1 subunit on T172, and the state of acetyl-CoA carboxylase (ACC) phosphorylation of S79 (the site of regulatory phosphorylation of ACC by AMPK [28]). Studies were done of each cell line in basal medium (Dulbecco's modified Eagle's medium with 10% fetal calf serum and penicillin/streptomycin), unless stated otherwise.

2.4.1. Measurement of isolated AMPK activity. The clonal PS120 lines were grown to confluency on 100 mm dishes. The cells were lysed in the presence of Triton X-100 lysis buffer, as described previously [5], and immunoprecipitated in the presence of Protein A/G Plus agarose (Santa Cruz) and either anti-α(2–20) or anti-HA antibody. Following adsorption, immunoprecipitates were washed and resuspended in buffer A (20 mM Tris–HCl, pH 7.4, 50 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM dithiothreitol, 1 mM PMSF, 2 μg/ml leupeptin, 25 μg/ml soybean trypsin inhibitor, and 50 μM benzamidine). The AMPK activity of resuspended immunoprecipitates was measured as previously described [5]. To determine any effects of residual AMP binding to heterotrimer, immunoprecipitated AMPK heterotrimer was incubated for 15 min at 30°C in the presence of 5′-nucleotidase (62 mU/ml) from *Crotalusa atrox* (Sigma). Following incubation, the nucleotidase was removed from the immobilized heterotrimer by washing several times in buffer A prior to resuspension and measurement of AMPK activity in the presence or absence of 200 μM AMP.

2.4.2. Assessment of ACC and AMPK phosphorylation state. Clonal lines expressing NHE, pMT2-HA-γ1 (and NHE) and pMT2-HA-R70Q-γ1 (and NHE) were incubated in the presence of 1 mM 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), or an equivalent volume of water, for 2 h at 37°C. Subsequently, the cells were lysed for 2 min in buffer A containing 0.4 mg/ml digitonin. Samples were precipitated with 0.54 volumes of saturated ammonium sulfate for 60 min on ice and centrifuged for 30 min at 14 000 rpm. The pellets were resuspended in buffer A and matched for protein prior to immunoblotting with horseradish peroxidase (HRP)-conjugated streptavidin to detect ACC or an ACC phospho-Ser79 specific antibody. To compare ACC phosphorylation with AMPK activation, the immunoblots were also probed with the anti-α(2–20) and the anti-phospho-T172 antibodies.

2.5. Miscellaneous

Protein content of samples was determined using the BCA assay (Pierce). Data are given as mean of n samples ± S.E.M., where n is the number of samples analyzed in each investigation. Comparisons between means were done using Student's t test for paired data using MYSTAT®.

3. Results

3.1. The CBS domains of the AMPK γ isoforms

A R200Q mutation in PRKAG3 has been demonstrated to be associated with increased skeletal muscle glycogen deposition [25]. This substitution is situated within the first of four CBS domains, present in the γ subunit. To determine the

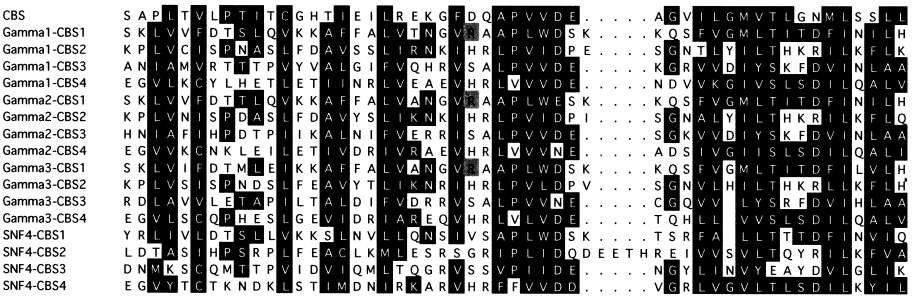

Fig. 1. Alignment of the CBS domains. Shown are the alignments of the CBS domains present in human CBS (GenBank™ accession number NP_000062) and the four present in human AMPK γ1 (U42412), human AMPK γ2 (AJ249976), human AMPK γ3 (AJ249977) and yeast Snf4p (M21760). The conserved arginine at position 70 in the AMPK γ1 subunit and the corresponding arginine residues in the γ2 and γ3 subunits are shaded gray. Identities or conservative amino acid substitutions are shaded black.

extent of conservation of this arginine residue, the CBS domains present in the human CBS, in the three human γ isoforms and in the yeast Snf4p were aligned (Fig. 1). Like the pig γ3 isoform, the human γ3 isoform also contains an Arg residue at the same position. Similarly, both human γ1 and γ2 demonstrate conservation of an Arg at this position. In contrast, neither the human CBS nor Snf4p possesses an Arg at this location. Interestingly, comparison of the human γ isoforms demonstrates that although the conserved Arg is only present in CBS-1 of this alignment, there are also Arg residues in juxta-positions in the remaining three CBS domains. In both CBS-2 and CBS-4 an arginine is present at the +1 position, whereas in CBS-3 an Arg is present at the −2 position in all three human γ isoforms. Furthermore, a second Arg is present at the −3 position in both γ2 and γ3. Similarly, Snf4p has conserved Arg residues at the +1, −2 and +1 positions for CBS domains 2, 3 and 4, respectively. Although the occurrence of these additional conserved Arg residues implies that they too may play a critical role in protein function, the aim of this study was to focus on the conserved Arg in the CBS-1 domain, given the observations in the Hampshire pig. We elected to study the γ1 isoform, the γ isoform with the widest tissue expression, to assess the impact of an R→Q mutation within the CBS-1 domain (R70Q) on AMPK activity.

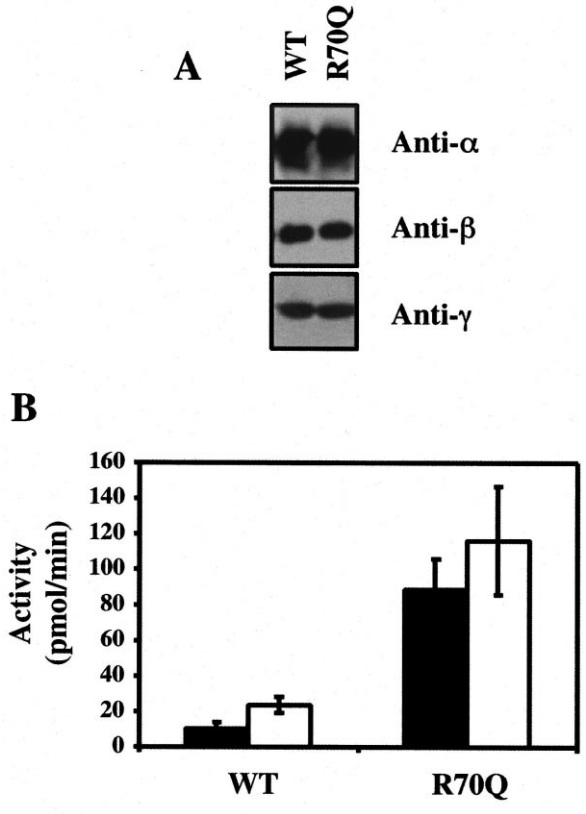

Fig. 2. Characterization of the R70Q γ1 mutation on AMPK heterotrimer expressed in COS7 cells. COS7 cells were co-transfected with vectors encoding either pMT2-HA-γ1 WT or R70Q mutant with pEBG-α1 and pBK-CMV-β1 with FUGENE® reagent as described in Section 2. Cells were harvested at 48 h post-transfection and glutathione adsorbates prepared from the lysates. In A, eluted WT and R70Q-containing heterotrimeric proteins were analyzed by immunoblot using anti-α (upper panel), anti-β (middle panel) or anti-γ (lower panel) antibodies, as described in Section 2. In B, WT and R70Q-containing heterotrimeric proteins eluted from glutathione adsorbates were assayed in the absence (black bars) or in the presence (open bars) of AMP (200 μM). Kinase activity of each construct is expressed as pmol of ^{32}P transferred to the SAMS peptide/min, in the presence of equivalent amounts of heterotrimer. Data presented as mean ± S.E.M. of $n=4$ enzyme isolates. The R70Q activity is statistically different from the WT activity at $P<0.001$ by a two-tailed t test.

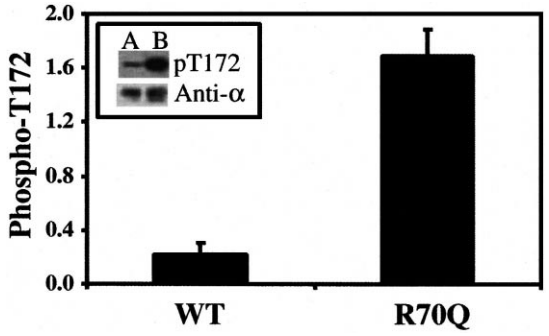

Fig. 3. Increased phosphorylation of the AMPK α subunit at Thr172 in R70Q γ1 heterotrimer. Inset: isolated WT (A) and R70Q (B) γ1-containing heterotrimeric proteins were probed with phospho-T172 specific or anti-α antibodies (upper and lower panels, respectively). Quantitation of the T172p immunoblot is represented as mean T172 phosphorylation in arbitrary units (± S.E.M. of $n=4$ isolates) corrected for α subunit content, as judged by densitometry of the immunoblots. The level of phosphorylation of T172 in the R70Q γ1-containing heterotrimers is statistically different from that of WT at $P<0.001$ by a two-tailed t test.

3.2. *R70Q γ1-containing AMPK heterotrimer expressed in COS cells*

In COS7 cells, we transiently expressed by triple transfection of cDNAs AMPK heterotrimers containing AMPK α1 and β1 and either WT γ1 or R70Q γ1. Heterotrimers were then isolated to homogeneity by glutathione adsorption chromatography for analysis. As shown by immunoblotting analysis, the R70Q γ1 mutation had no apparent impact on the total of expressed enzyme or on the composition of the AMPK heterotrimer obtained with equivalent amounts of α, β and γ in each (Fig. 2A). Measurement of AMPK activity in the presence and absence of AMP revealed two major changes associated with the R70Q γ1-containing heterotrimer (Fig. 2B). First, the mutant was activated 5–9-fold when compared to the WT heterotrimer. Second, the mutant had markedly reduced AMP dependence; the WT enzyme basal activity in the absence of AMP is 45% of the total measured in the presence of AMP, while the mutant enzyme's AMP-independent activity rises to 77% of the total.

Since the phosphorylation of T172 on the α subunit is required for enzyme activity [5–8], we postulated that a higher fraction of the R70Q γ1-containing heterotrimers were phosphorylated on this residue. This prediction was confirmed on immunoblotting of the isolated heterotrimers with an antibody specific for the T172p epitope (Fig. 3). As determined by the analysis of several such isolates, there is a greater than 8-fold increase in the phosphorylation of R70Q γ1-containing heterotrimers at T172. Taken together, these data indicate that R70Q γ1 mutation allows the accumulation of active AMPK enzyme under conditions where there is much less activation of the WT enzyme.

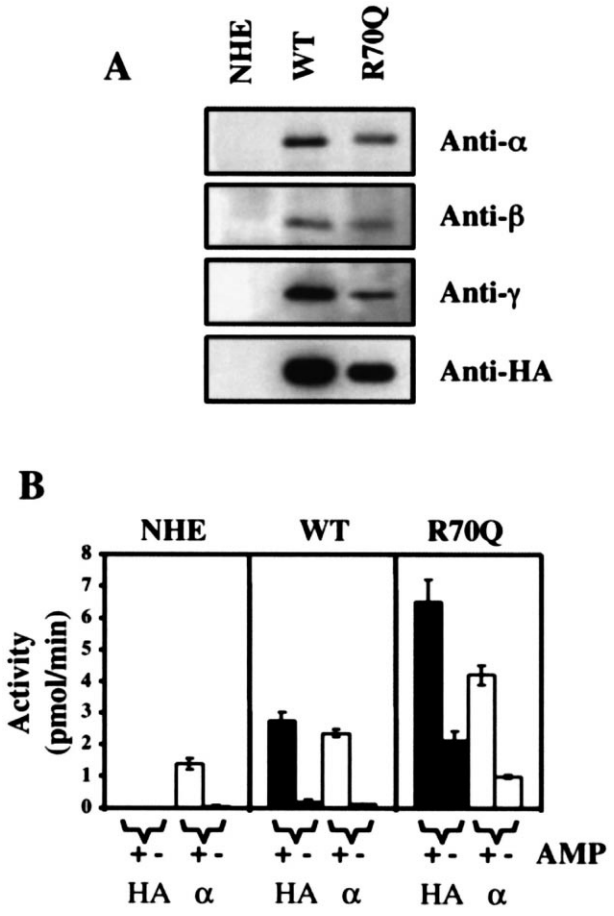

Fig. 4. Stable expression and characterization of WT and R70Q γ1 subunit in a PS120 cell line. The vector pECE-NHE, conferring acid resistance, was transfected into the lung fibroblast PS120 cell line in the presence or absence of pMT2-HA-γ1 or pMT2-HA-R70Q-γ1. Following acid selection and cell subcloning as described in Section 2, confluent cells were lysed and AMPK heterotrimer immunoprecipitated with anti-HA antibody prior to immunoblotting. In A, AMPK subunits were blotted with anti-α, -β, -γ and -HA specific antibodies to confirm the association of transfected WT and mutant γ subunit with the endogenous α and β subunits. In B, the WT γ1, R70Q γ1 and control PS120 stable cell lines were grown to 90% confluency in 6-well plates. Cells were lysed and AMPK heterotrimer immunoprecipitated from equal amounts of lysate protein with either anti-α(2–20) antibody (open bars) or an anti-HA specific antibody (black bars). AMPK activity was assayed in the presence (+) or in the absence (−) of 200 μM AMP. Kinase activity of each construct is expressed as pmol of ^{32}P transferred to the SAMS peptide/min. Data presented as mean ± S.E.M. of $n = 3$ experiments. The activity in the R70Q γ1-containing heterotrimers in all instances is significantly different from the WT values at $P < 0.005$ by two-tailed t test.

3.3. Stable expression of R70Q γ1-containing AMPK heterotrimer

To investigate the effect of chronic expression of the R70Q γ1, we generated clonal PS120 cell lines stably overexpressing the WT or mutant (R70Q) γ1 subunits. As a control line, we employed PS120 cells stably expressing the selection vector pECE-NHE3. Immunoprecipitation of cell lysates with anti-HA antibody was successful in capturing AMPK heterotrimer containing α, β and HA-tagged γ1 (Fig. 4A). These data indicate that the plasmid-expressed γ subunits can recombine with endogenous β and α subunits and that the R70Q mutation did not influence this association. Immunoblotting of cell lysates with α isoform-specific antibodies revealed only the α1 isoform in these cells (data not shown). Precipitation of AMPK heterotrimers with anti-HA antibody confirms a 2.5-fold stimulation of the activity of the R70Q γ1-containing heterotrimers measured in the presence of AMP (Fig. 4B). Furthermore, the R70Q γ1-containing heterotrimer displayed 30% of maximal activity in the absence of AMP, while the WT γ1-containing heterotrimer was nearly inactive. Nearly identical data were obtained on analysis of AMPK immunoprecipitated with the anti-α(2–20) antibody. Comparison of samples immunoprecipitated with anti-HA antibody and that with anti-α(2–20) antibody did indicate that more activity was isolated from both WT and mutant lines with the anti-HA antibody. This difference in the isolated activities may be representative of the differing affinities of each antibody for heterotrimer. Alternatively, this may suggest that there is more AMPK activity present in these cell lines than is recovered by the α1/α2 recognizing pan antibody or possibly interference of this α antibody with the activity of the catalytic α subunit. The increased expression of AMP-independent activity of the R70Q γ1-containing heterotrimers does not appear to be a function of tightly bound AMP persisting through the immunoisolation. Treatment of immunoprecipitates with 5′-nucleotidase, a technique shown by others to diminish the activity of isolated enzyme by hydrolysis of bound AMP [6], had no effect on this AMP-independent activity (data not shown).

To determine if the increase in AMPK activity of the stably expressing cell lines was due to increased phosphorylation of the α subunit at T172, as observed in the transiently expressed AMPK, immunoblots were performed of cell extracts after incubation of cells in the presence or absence of the AMPK activator, AICAR. AICAR, following its metabolism to ZMP, normally causes activation and phosphorylation of AMPK α on T172 [29,30]. Immunoblotting with anti-

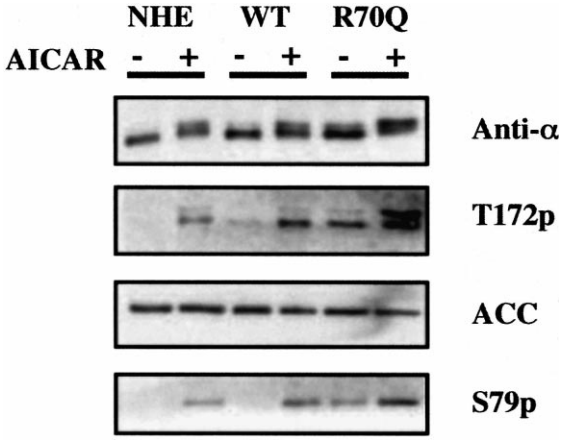

Fig. 5. Stimulation of stable cell lines demonstrating basal activation of the R70Q heterotrimer. WT γ1, R70Q γ1 and control (NHE) PS120 stable cell lines were grown to 90% confluency in 6-well plates. Cells were treated in the absence (−) or presence (+) of 1 mM AICAR for 2 h prior to digitonin lysis and ammonium sulfate precipitation. In this representative experiment, resuspended protein was immunoblotted and the membranes were probed with anti-AMPK α(2–20) (upper panel), anti-AMPK phospho-T172 (second panel), HRP-conjugated streptavidin (to detect total ACC) (third panel) and anti-ACC phospho-Ser79 (fourth panel) specific antibodies.

α(2–20) antibody demonstrates, as expected, a shift in the mobility of the α subunits in NHE control line and the WT γ1-expressing lines following AICAR treatment (Fig. 5, upper panel). To confirm that phosphorylation of T172 was, in part, responsible for the mobility shift, extracts were also blotted with the phospho-T172 specific antibody, which confirmed this AICAR-stimulated phosphorylation (Fig. 5, second panel). As can be seen in Fig. 5 (the two upper most panels), the overexpression of R70Q γ1 results in an α subunit that is already phosphorylated with a small shift in gel mobility in the absence of AICAR stimulation. The addition of AICAR causes an increase in the gel shift and increased phosphorylation of a second slower migrating band, perhaps suggesting another site of AICAR-stimulated phosphorylation causing additional charge heterogeneity.

To determine if the stimulated AMPK activities seen in the lysates and immunoprecipitates derived from the overexpressing R70Q γ1 cells were reflective of heightened intracellular activity, cell extracts were examined for the state of ACC phosphorylation, as one of the major targets of AMPK. Overexpression of either WT or R70Q γ1 in PS120 cells had no effect on overall ACC expression, as determined by streptavidin–HRP blotting (Fig. 5, panel three). Immunoblotting with ACC isoform-specific antibodies reveals that only ACC-α is expressed in these cells (data not shown). Blotting of extracts with an antibody directed against the major regulatory phosphorylation site for AMPK (Ser79p) on ACC showed no phosphorylation of ACC in basal cells, but an increase in ACC Ser79 phosphorylation in response to AICAR in control and WT γ cell lines (Fig. 5, lower panel), mirroring the observed changes in AMPK Thr172 phosphorylation/activation. In extracts derived from the overexpressing R70Q γ1 cells, ACC is already phosphorylated at Ser79 with a small increase on the addition of AICAR, consistent with the increase in basal AMPK activity seen in immunoprecipitates and the heightened T172 phosphorylation of the α subunit in extracts.

Immunolocalization of the PS120 cells expressing WT γ1 and R70Q γ1 demonstrated no significant difference in the cellular distribution of either the HA-tagged γ1 subunits or of endogenous α subunits (α1 and α2) (data not shown). In these cell lines, the predominant expression of both γ and α subunits is largely nuclear, but with a substantial amount of extranuclear localization as well. Electron microscopy of the control NHE, WT γ1 and R70Q γ1-expressing cell lines did not indicate any ultrastructural differences between them (data not shown).

In preliminary studies, we have begun to examine other features of the cellular phenotype of PS120 cells, a pulmonary fibroblast line. The overexpression of WT or R70Q γ1 in these cells has no effect on the rate of glycogen synthesis, glucose transport (in the presence or absence of cytochalasin B) or on cell proliferation (data not shown).

4. Discussion

The present investigation characterizes the functional consequence of an Arg to Gln substitution within the first CBS domain of the human AMPK γ1 subunit. The basis of this mutation derives from a report on a homologous mutation in the AMPK γ3 isoform isolated from pig skeletal muscle [25]. Sequence analysis of the three human γ isoforms indicates that this Arg residue is conserved at an equivalent position in CBS-1 of each protein, prompting our investigation. The γ3 isoform is rather restricted in its tissue expression. Northern blot analysis has demonstrated a predominance in skeletal muscle, while immunoprecipitation with anti-γ3 antibody showed AMPK activity primarily in the brain and testis [16]. In contrast, both γ1 and γ2 are widely expressed, with γ1 being present in most tissues.

Our results demonstrate that the R70Q γ1 mutation results in an increase in AMPK activity during acute and chronic expression. Under acute conditions, we observed a 5–9-fold increase in activity, while chronic expression demonstrated a 2–3-fold increase in activity. In both models, there was a striking increase in the activity of AMPK in the absence of AMP. Furthermore, in both instances, the increase in activity was paralleled by a concurrent increase in basal phosphorylation of the AMPK α subunit at the site of activating phosphorylation by AMPKK(s) [5–8]. The impact of the γ3 subunit mutation at the equivalent position seemed to indicate a decrease in AMPK activity, as measured in crude muscle extracts, though the authors speculated that, in fact, there had been an initial increase in kinase activity resulting in glycogen accumulation, followed by inhibition of kinase activity subsequent to this fuel storage [25]. Our study does not clarify the impact of the Hampshire pig mutation on the phenotype observed in the pig skeletal muscle, as we have studied a different γ subunit and, in the cells employed, have observed no changes in glucose transport or glycogen synthesis.

The mechanism by which the mutation in γ1 activates AMPK activity cannot be totally clarified from the present study, but some features are notable. The mutant heterotrimer appears to have increased AMP-independent activity in both model systems studied. The binding site for AMP on the enzyme heterotrimer has not been identified and, other than the catalytic ATP binding site on the α subunit, there are no characteristic adenylate binding sites on any of the individual subunits, suggesting that higher order structure contributed to by all three subunits underlies AMP stimulation. The γ subunit has been shown to influence the degree of AMP stimulation of the reconstituted heterotrimer with the γ3-containing heterotrimer showing the least degree of AMP stimulation [16]. Perhaps a structural change as a result of the γ mutations mimics structurally the interactions of AMP with sequences on the γ isoform, causing AMP-like activation. We have noted that the mutation is associated with heightened phosphorylation of the activation loop T172 on the α subunit. The phosphorylation of this residue by upstream AMPKKs has been shown to be stimulated and its dephosphorylation by protein phosphatases inhibited by binding of AMP to the heterotrimer [6,7]. It is tempting to speculate that the γ1 mutation, by being 'AMP-mimetic', causes a similar change in the reactivity of the heterotrimer with AMPKK(s) or protein phosphatases, resulting in increased phosphorylation and activation of the heterotrimer. Alternatively, it is possible that the degree of antecedent T172 phosphorylation influences the interaction of the heterotrimer with AMP.

The activating effect of the γ1 mutation is not just observed in vitro with measured AMPK activity, but also in intact cells. ACC is one of the major substrates for AMPK and is phosphorylated on Ser79 (and other residues) in response to AMPK [28]. In our experiments, we were able to document the ability of AICAR to stimulate phosphorylation of ACC Ser79 in control (NHE) and WT γ1-overexpressing cells,

although in the basal state, no ACC phosphorylation at this site was detectable. In contrast, ACC Ser79 is already nearly maximally phosphorylated in the absence of AICAR in R70Q γ1-overexpressing cells, consistent with maximal AMP-independent activity within the cell. AICAR, which activates the enzyme after its conversion to ZMP, an AMP-mimetic nucleotide, has only a small additional effect on ACC phosphorylation, consistent with the AMP-independent nature of the activation of the intracellular enzyme.

The position of the Arg to Gln mutations in CBS-1 of the AMPK γ subunits is equivalent to the reported D444N mutation in the CBS domain of CBS [24]. This latter mutation has no effect on basal catalytic activity, but does abolish the activation of CBS by *S*-adenosylmethionine. Taken together, these data suggest that the CBS domain may play a regulatory role for both AMPK and CBS by influencing interactions with adenosine derivatives. An attempt to clarify if AMP was interacting directly with the CBS domain of the AMPK γ subunit has been addressed [16]. This study, using two different adenosine derivatives, was unable to show specific binding to the γ subunit alone, but did show that saturating concentrations of AMP did have a protective effect against the binding of 8-azido-[^{32}P]AMP. Therefore, the exact role of the CBS domain remains elusive, but the present study confirms the importance of the residues at equivalent positions to Arg70 of AMPK γ1 in this and other CBS domain-containing proteins.

Note added in proof

After acceptance of this manuscript, Gollob and co-workers reported that an autosomal dominant R302Q mutation in the first CBS domain of the γ2 subunit (a position equivalent to R70 in γ1) is responsible for familial Wolff-Parkinson-White syndrome, the second most common cause of cardiac paroxysmal supraventicular tachycardia (*New England Journal of Medicine*, 344, 1823–1831, 2001). While the author's do not report the effects of the R302Q mutation on enzyme activity, it seems likely, based on our investigation, that enhanced AMPK activity is responsible for the developmental and functional defects in cardiac conduction in this syndrome.

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