AMP-activated protein kinase phosphorylates and inactivates liver glycogen synthase

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Recombinant muscle GYS1 (glycogen synthase 1) and recombinant liver GYS2 were phosphorylated by recombinant AMPK (AMP-activated protein kinase) in a time-dependent manner and to a similar stoichiometry. The phosphorylation site in GYS2 was identified as Ser7, which lies in a favourable consensus for phosphorylation by AMPK. Phosphorylation of GYS1 or GYS2 by AMPK led to enzyme inactivation by decreasing the affinity for both UDP-Glc (UDP-glucose) [assayed in the absence of Glc-6-P (glucose-6-phosphate)] and Glc-6-P (assayed at low UDP-Glc concentrations). Incubation of freshly isolated rat hepatocytes with the pharmacological AMPK activators AICA riboside (5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside) or A769662 led to persistent GYS inactivation and Ser7 phosphorylation, whereas inactivation by glucagon treatment was transient. In hepatocytes from mice harbouring a liver-specific deletion of the AMPK catalytic α1/α2 subunits, GYS2 inactivation by AICA riboside and A769662 was blunted, whereas inactivation by glucagon was unaffected. The results suggest that GYS inactivation by AMPK activators in hepatocytes is due to GYS2 Ser7 phosphorylation.

Key words: A769662, 5-aminoimidazole-4-carboxamide-1-beta-D-ribofuranoside (AICA riboside), AMP-activated protein kinase (AMPK), glucagon, glycogen synthase, hepatocyte.

INTRODUCTION

Glycogen is a branched homopolymer made of glucosyl units and is essential for whole-body glucose homoeostasis. During hyperglycaemia, glucose is incorporated into glycogen by the combined action of GYS (glycogen synthase) and glycogen branching enzyme, which catalyse α-1,4- and α-1,6-glycosidic bond formation respectively [1]. In mammals, a muscle isoenzyme (GYS1) is ubiquitously expressed, but in liver the hepatic iso-enzyme (GYS2) is predominantly expressed [1]. These isoenzymes are 70% identical and possess a highly conserved catalytic central region flanked by two variable extremities [2].

The rate of incorporation of glucose into liver glycogen directly correlates with GYS activity, which is regulated by allosteric and phosphorylation/dephosphorylation mechanisms [2–4]. Several protein kinases inactivate GYS by phosphorylation. However, GYS inactivation, at least in skeletal muscle, can be overcome to some extent by Glc-6-P (glucose 6-phosphate) allosteric activator, whose intracellular concentration rises when glucose transport is increased [5,6]. A total of nine different phosphorylation sites are found in the three domains of GYS1, seven of which are conserved in GYS2 [2,7]. The N-terminal domain contains site 2 (Ser7) in GYS1 phosphorylated by different protein kinases, including PKA (cAMP-dependent protein kinase), PhK (phosphorylase kinase) and AMPK (AMP-activated protein kinase), leading to GYS inactivation [8,9]. Site 2 phosphorylation allows site 2a (Ser10) phosphorylation by phosphorylation by the constitutively active CK1 (casein kinase 1), resulting in further and substantial enzyme inactivation [10]. The central domain contains sites 3a (Ser640), 3b (Ser644), 3c (Ser648), 4 (Ser650) and 5 (Ser656), whose phosphorylation is hierarchical and also inactivates GYS [7]. Phosphorylation at site 5 by CK2 allows sequential phosphorylation of the other sites by GSK3 (glycogen synthase kinase 3). In addition, sites 3a and 3b can be phosphorylated directly by other kinases [11–13]. The C-terminal domain, which is absent in GYS2, contains site 1a (Ser656) phosphorylated by PKA, and site 1b (Ser658) phosphorylated by Ca2+/calmodulin-dependent protein kinase II [2,14]. The relative importance of each site in the control of GYS1 activity has been studied by site-directed mutagenesis, revealing a crucial role for site 2, 2a, 3a and 3b phosphorylation for the control of GYS1 expressed in COS cells [15]. For GYS2, on the other hand, mutation of Ser7 to an alanine residue suffices to fully activate the enzyme expressed in FTO2B cells [16], suggesting that phosphorylation at Ser7 might be more important for GYS2 inactivation than phosphorylation at the other conserved GSK3/CK1/CK2 sites. One candidate kinase for phosphorylation of GYS2 at Ser7 is AMPK, known to phosphorylate GYS1 site 2.
in vivo [8,17,18]. Moreover, this site lies in a favourable consensus for AMPK phosphorylation in GYS2.

AMPK is a heterotrimeric protein kinase composed of a catalytic α-subunit and two regulatory subunits (β and γ) [19–21]. Each subunit has different isoforms allowing the expression of 12 different complexes, excluding splice variants. AMPK activation occurs during metabolic stress and requires activation loop Thr172 phosphorylation of its α-catalytic subunits by upstream kinases. Once activated, AMPK stimulates ATP-producing pathways and inhibits anabolic processes to maintain intracellular energy status [19,20]. AMPK activation also influences whole-body energy homoeostasis through appetite control and re-orientation of metabolism [19,21]. Important influences whole-body energy homoeostasis through appetite intracellular energy status [19,20]. AMPK activation also has long-term effects by repressing lipogenic gene expression of 12 different complexes, excluding splice variants.

Ser7 phosphorylation in freshly isolated rat hepatocytes and that by pharmacological activators leads to GYS inactivation and has been genetically deleted. GYS inactivation by the compounds was blunted in incubated hepatocytes from mice whose two AMPK catalytic subunits had been genetically deleted.

Recombinant GYS1 and GYS2 were purified as described previously [5]. Briefly, HEK (human embryonic kidney)-293 cells were co-transfected with expression vectors for untagged GYS1 or GYS2 and GST (glutathione transferase)-tagged glycogenin. After 36 h, the cells were lysed and GYS–GST–glycogenin complexes were purified by chromatography on glutathione-Sepharose with elution by glutathione. Recombinant bacterially expressed α,β,γ, AMPK was fully activated by incubation with recombinant bacterially expressed LKB1 (liver kinase B1)–Mo25–STARD (STE20-related kinase adapter protein) complex, both kindly provided by Dr Diethelm Neumann (ETH Zurich, Switzerland), as described previously [30] except that LKB1 was used instead of calmodulin-dependent protein kinase-β. PKA catalytic subunits were purified from bovine heart as described previously [31]. Bovine hearts were obtained from the local slaughterhouse. Recombinant CK1 was from New England Biolabs. PP1c–GST (PP1c is protein phosphatase 1γ catalytic subunit) was purified using GSTrap™ HP columns (GE Healthcare) from a lysate of Escherichia coli BL21 bacteria transformed with pGEX-PP1c, and cultured in the presence of 1 mM MnCl₂.

Experiments involving the preparation of hepatocytes from anaesthetized rats and mice were approved by the local animal ethics committee.

**Experimental**

**Materials**

AICA riboside (Toronto Research Chemicals) and glucagon were from Novo Nordisk. A769662 was kindly provided by Dr Anudharan Baladran (AstraZeneca, Mölndal, Sweden). The AMARA [28] and MR6 [29] peptides were kindly synthesized by Dr Vincent Stroobant (Ludwig Institute, Brussels, Belgium). Anti-(p-Thr172 AMPK α-subunit), anti-(p-Ser79 ACC1 (acetyl-CoA carboxylase 1)) (Millipore) and anti-[total eEF2 (eukaryotic elongation factor-2)] (Santa Cruz Biotechnology) antibodies were from the sources indicated. Anti-(total AMPK α1/α2), anti-(total GYS1) and anti-(p-Ser7 GYS1) antibodies were kindly provided by Professor Grahame Hardie (College of Life Sciences, University of Dundee, Dundee, Scotland, U.K.). Anti-GYS2 and anti-(p-Ser74 GP (glycoprotein phosphorylase)] antibodies [5] were from the MRC Protein Phosphorylation Unit (University of Dundee, Dundee, Scotland, U.K.). Anti-(p-Ser2 GYS2) was raised in rabbit as described previously [16], and anti-(sheep IgG coupled to horseradish peroxidase), anti-(total GP) (Sigma) and anti-(rabbit IgG coupled to horseradish peroxidase) (GE Healthcare) antibodies were from the sources indicated.

Recombinant GYS proteins (2.5 μg) were first dephosphorylated by incubating with 0.7 μg of purified recombinant PP1c–GST in 30 μl of dephosphorylation buffer (50 mM Hepes, pH 7.5, 1 mM MnCl₂, 0.1 mM EGTA and 0.1 % 2-mercaptoethanol) for 30 min at 30°C and the reactions were stopped by adding 150 nM microcystin-LR (Sigma). The preparations were then phosphorylated with 200 μM units of AMPK or 200 μM units of PKA in 60 μl of reaction mixture containing 25 mM Hepes, pH 7.5, 0.5 mM MnCl₂, 50 μM EGTA, 0.05 % 2-mercaptoethanol, 200 μM AMP, 10 mM MgCl₂, 175 nM microcystin and 0.1 mM [γ-32P]MgATP (specific radioactivity of 1000 c.p.m./pmol) at 30°C. Aliquots (10 μl) were removed at the times indicated in the Figure legends and stopped in SDS/PAGE sample buffer [100 mM Tris/HCl, pH 6.8, 10 % (v/v) glycerol, 5 % (v/v) 2-mercaptoethanol, 8 % (w/v) SDS and 0.025 % Bromophenol Blue]. Proteins were separated by SDS/PAGE (10 % gels) for staining with PAGEBlue™ (Thermo Scientific). GYS protein was quantified by gel infrared imaging (LI-COR Odyssey™) along with known amounts of rabbit aldolase whose concentration had been determined from the calculated molar absorption coefficient and the measured absorbance at 280 nm [30]. For determination of 32P incorporation, GYS bands were excised from the gel, dissolved in 500 μl of 3 % (w/v) H₂O₂ (Sigma) by heating for 2 h at 80°C, mixed with 5 ml of scintillant (Ultima Gold™; PerkinElmer) and radioactivity was counted. Stoichiometries of phosphorylation were expressed as mol of 32P incorporated/mol of GYS using the calculated molecular mass of each isoenzyme.
Analysis of phosphorylated recombinant GYS preparations by MS

Recombinant GYS1 and GYS2 proteins (1.5 μg) were dephosphorylated then phosphorylated, using non-radioactive MgATP, by PKA and AMPK as described above. Following SDS/PAGE and in-gel trypsin digestion, peptides were analysed by static nanoelectrospray ionization–tandem MS in an LTQ XL ion-trap mass spectrometer (ThermoFisher Scientific) and the phosphorylation sites were pinpointed using an MS²/MS³ neutral loss method. For quantification by SRM (single reaction monitoring), tryptic peptides were analysed by LC–tandem MS with the LTQ XL equipped with a microflow ESI source interfaced to a Dionex Ultimate Plus Dual gradient pump, a Switchos column switching device and a FAMOS Autosampler (Dionex).

Separation was on a BioBasic C18 column (180 μm × 15 cm) (ThermoFisher Scientific) equilibrated in solvent A [5% (v/v) acetonitrile and 0.05 % formic acid in water] using a 90 min gradient from 0 to 70 % solvent B [80% (v/v) acetonitrile and 0.05 % formic acid in water] at a flow rate of 1.5 μl/min. The mass spectrometer was operated in a data-dependent manner to follow up four SRM transitions corresponding to mono-, di- and un-phosphorylated forms of the Ser7 and Ser⁵⁰ GYS2 peptide and an internal tryptic peptide from GYS2 as a reference. Abundances of each molecular species were determined by peak area intensity integration.

GYS assay

The recombinant GYS1/2 preparations were dephosphorylated then rephosphorylated by AMPK or PKA, with or without CK1 (5 units to ensure maximal inactivation) as described above using non-radioactive MgATP. The reaction mixtures were placed on ice for the GYS assay using a method of the modified [33] by following the incorporation of UDP-[U-¹⁴C]-d-Glc (PerkinElmer) into glycogen. Briefly, to determine the \( K_m \) for UDP-Glc (UDP-glucose), 100 ng of GYS was incubated in 100 μl of assay buffer containing 25 mM Hepes, pH 7.8, 50 mM NaF, 1 mM EDTA, 0.7 % shellfish glycogen and 0, 0.05, 0.1, 0.5, 1, 2.5 or 4.6 mM UDP-[U-¹⁴C]-d-Glc with or without 10 mM Glc-6-P. To determine the \( K_v \) for Glc-6-P, GYS2 was incubated as described above in the presence of 0.25 mM UDP-[U-¹⁴C]-d-Glc and 0, 0.01, 0.02, 0.05, 0.125 or 0.25 mM Glc-6-P. The \( K_v \) of GYS1 for Glc-6-P was measured with 0.1 mM UDP-[U-¹⁴C]-d-Glc and 0, 0.02, 0.05, 0.125, 0.25 or 1 mM Glc-6-P. The reactions were stopped by spotting 90 μl aliquots on to 2 cm × 2 cm Whatman 31 ET CHR papers (GE Healthcare), which were plunged into ice-cold 66 % (v/v) ethanol. After washing in ethanol and a final wash in acetone, the dried papers were transferred to vials for scintillation counting. The kinetic parameters \( V_{max} \), \( K_m \) for UDP-Glc and \( K_v \) for Glc-6-P were obtained by non-linear curve fitting using GraphPad Prism.

**AMPK\textsubscript{α1,2LS}\textsuperscript{−/−} mice**

LS (liver-specific) AMPK\(\alpha_2\)−/− mice were obtained by crossing floxed AMPK\(\alpha_2\) mice with mice expressing Cre recombinase under the control of both the mouse albumin regulatory elements and the \( α\)-fetoprotein enhancers. AMPK\(\text{α1,2LS}\textsuperscript{−/−}\) mice were then bred by crossing these mice with AMPK\(\text{α1}\textsuperscript{−/−}\) mice [34].

Preparation and incubation of freshly isolated rat and mouse hepatocytes

Freshly isolated hepatocytes from anaesthetized overnight-starved male Wistar rats (approximately 200 g of body weight) were prepared by perfusion with collagenase as described previously [35]. A scaled-down procedure was also used to prepare isolated cells from starved WT (wild-type) and AMPK\(\text{α1,2LS}\textsuperscript{−/−}\) mice (approximately 30 g of body weight). Hepatocytes (50 mg of wet weight/ml) were gassed with O\(_2\)/CO\(_2\) (19:1) and pre-incubated for 40 min in Krebs–Henseleit buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl\(_2\), 1.18 mM KH\(_2\)PO\(_4\), 1.18 mM MgSO\(_4\) and 25 mM NaHCO\(_3\), pH 7.4) with 30 mM glucose, 5 mM glutamine and 5 mM leucine at 37°C to fully activate GYS [36]. Agonists were then added to the incubation medium at final concentrations of 1 mM AICA riboside, 30 μM A769662 (from a stock solution in DMSO) or 10⁻¹ M glucagon (control incubations without A769662 received 0.1 % DMSO).

Duplicate samples were taken at 0, 5, 15 and 30 min. For GYS assay, 250 μl of cell suspension was removed, mixed with 50 μl of ice-cold buffer containing 120 mM glycylglycine, pH 7.4, 75 mM KF, 7 mM EDTA and 0.5 % shellfish glycogen and immediately frozen in acetone/solid CO\(_2\). Thawed extracts were centrifuged and aliquots (25 μl) were assayed for GYS as described above with 2.5 mM UDP-Glc, but in the absence of Glc-6-P and in the presence of 9.3 mM Na\(_2\)SO\(_4\) [37]. In some experiments, GP was assayed as described previously [37]. For AMPK assays and immunoblotting, cells were harvested by rapidly centrifuging 250 μl of cell suspension (Beckman bench MicroFuge B; 20 s at 9500 g). After removing supernatants, the cell pellets were immediately frozen in liquid nitrogen for storage at −80°C. Extracts were prepared and precipitated with 10 % (w/v) poly(ethylene glycol) 6000 for AMPK assays and immunoblotting [32].

Immunoblotting

Extracts from rat and mouse hepatocytes (50 μg of protein) were subjected to SDS/PAGE. Anti-(rabbit IgG) conjugated to IR dye 800 (Rockland) and anti-(sheep IgG) conjugated to Alexa Fluor® 680 (Molecular Probes) were used to measure the phosphorylation state of AMPK and ACC by LI-COR Odyssey™ infrared imaging as described previously [32]. The phosphorylation states of GYS2 Ser7 and GP Ser14 were measured by chemiluminescence as described previously [32], and equal sample loading was verified by blotting for full-length eEF2.

Other methods

Protein was estimated by the method of Bradford [38] with γ-globulin as a standard. The results are expressed as means ± S.E.M. for the indicated number of individual experiments. The statistical significance of the results was assessed using a paired two-sided Student’s t test.

RESULTS

**AMPK phosphorylates Ser\(\alpha\) of GYS2**

GYS1 site 2 (Ser\(\alpha\)) is phosphorylated by AMPK in vitro [8] and in AICA riboside-treated and contracting muscles [18,39,40]. The corresponding site in GYS2 (Ser\(\alpha\)) also lies in a favourable consensus for phosphorylation by AMPK (Figure 1A). We first compared the time course of phosphorylation of recombinant GYS1 by equal amounts of AMPK or PKA catalytic subunits in terms of activity units measured towards substrate peptides. After 40 min of incubation, when the stoichiometry of \(^{32}\)P incorporation was maximal, phosphorylation by PKA was approximately 50 % higher than that observed with AMPK, reaching 0.46 ± 0.03 compared with 0.31 ± 0.02 mol of \(^{32}\)P incorporated/mol of GYS1 (Figure 1B). This result is consistent with the fact that...
there are two sites for PKA and one AMPK site described for the muscle GYS1 isoenzyme [7,8]. Like GYS1, GYS2 was also phosphorylated in a time-dependent manner by PKA and AMPK, but to a similar extent (maximal stoichiometry of 0.29 ± 0.02 and 0.25 ± 0.02 mol of 32P incorporated/mol of GYS2 respectively; Figure 1C). For PKA, only one phosphorylation site in GYS2 has been reported [2] and the comparable stoichiometry of phosphorylation of GYS1 and GYS2 by AMPK (Figure 1C) would suggest the presence of a single AMPK phosphorylation site in GYS2. Overall the stoichiometries of phosphorylation of the two GYS preparations were low, possibly because only a proportion of recombinant protein was in the proper conformation for phosphorylation by protein kinases.

In order to identify the site phosphorylated by AMPK in GYS2, recombinant GYS1 and GYS2 preparations were phosphorylated with non-radioactive ATP and either AMPK or PKA under conditions required to reach maximal phosphorylation. After SDS/PAGE and in-gel trypsin digestion, the samples were analysed by tandem MS and SRM. Only the single-phosphorylated tryptic peptide containing Ser7 was detected in AMPK- or PKA-phosphorylated GYS1 and GYS2 by SRM analysis. Tandem MS analysis of the peptide identified Ser7 as the residue phosphorylated by AMPK and PKA in the GYS isoenzyme preparations. Moreover, by SRM, only the singly phosphorylated peptide was detected with no indication of phosphorylation at Ser10. Immunoblotting of recombinant GYS1 and GYS2 phosphorylated by PKA and AMPK with a phospho-specific antibody raised against phosphorylated Ser7 for each isoenzyme confirmed that this site was phosphorylated by the two kinases (Figures 1D and 1E). Taken together, the results demonstrate that AMPK phosphorylates GYS2 at Ser7 in vitro.

Effect of phosphorylation by AMPK and PKA on the kinetic properties of GYS1 and GYS2

The recombinant GYS1 and GYS2 preparations were phosphorylated with non-radioactive ATP and either AMPK or PKA under conditions required to reach maximal phosphorylation. GYS was then assayed in the presence or absence of 10 mM Glc-6-P, a positive allosteric effector of GYS. The ratio of GYS activity measured with and without Glc-6-P provides an activity ratio to assess GYS inactivation induced by phosphorylation. After phosphorylation by AMPK or PKA, GYS1 was inactivated compared with non-phosphorylated control GYS1 assayed at low UDP-Glc concentrations (Figure 2A), but the effect was opposed at high UDP-Glc. AMPK- and PKA-induced GYS1 inactivation was also reflected by a decreased sensitivity towards stimulation of activity by Glc-6-P when assayed at low UDP-Glc (Figure 2B). In addition, at low UDP-Glc concentrations (0.05–0.5 mM) within the physiological range, a significant decrease (61 to 27% and 64 to 32%) in GYS2 activity ratio was observed after phosphorylation by AMPK or PKA respectively, which was counteracted by high UDP-Glc concentrations (2.5 and 4.6 mM) (Figure 2C). In addition, AMPK- and PKA-induced phosphorylation decreased the affinity of GYS2 for Glc-6-P when measured at low UDP-Glc (Figure 2D).
AMPK inactivation of liver glycogen synthase

GYS1 or GYS2 phosphorylation by AMPK or PKA had no significant effect on \( V_{\text{max}} \) in the presence or absence of Glc-6-P (Table 1). In addition, there was no significant effect of phosphorylation of the GYS isoenzymes by AMPK or PKA on the \( K_m \) for UDP-Glc measured in the presence of Glc-6-P (Table 1). However, when GYS2 was assayed in the absence of Glc-6-P, phosphorylation by AMPK or PKA increased the \( K_m \) for UDP-Glc from 0.39 mM to 0.70 and 0.88 mM respectively, and similar effects of phosphorylation were seen for GYS1. Phosphorylation of GYS1 and GYS2 by AMPK and PKA increased the \( K_a \) for Glc-6-P when measured at low UDP-Glc (Table 1). Thus phosphorylation of both GYS1 and GYS2 by AMPK or PKA leads to GYS inactivation by decreasing the affinity for both UDP-Glc (in the absence of Glc-6-P) and Glc-6-P (at low UDP-Glc concentrations). After phosphorylation by combinations of PKA/CK1 and AMPK/CK1 for GYS assay under identical conditions with those used to measure GYS in intact cells with 2.5 mM UDP-Glc, a 60% decrease in GYS2 activity ratio was observed (Figure 2E).

AMPK activation leads to Ser\(^7\) phosphorylation and inactivation of GYS in freshly isolated rat hepatocytes

Freshly isolated hepatocytes from overnight-starved rats were incubated with two different pharmacological AMPK activators (A769662 or AICA riboside) and with glucagon, which
inactivates liver GYS via PKA-induced phosphorylation. AMPK activation was assessed by measuring AMPK activity, AMPK Thr172 phosphorylation and phosphorylation of ACC Ser79, its best known downstream target. Treatment with AICA riboside induced an increase in AMPK activity, AMPK Thr172 phosphorylation and ACC Ser79 phosphorylation, which persisted for up to 30 min of incubation (Figures 3A, 3D and 3E). Treatment with A769662 caused no significant increase in AMPK activity (Figure 3A), but transiently increased AMPK Thr172 (Figure 3D) with A769662 caused no significant increase in AMPK activity for up to 30 min of incubation (Figures 3A, 3D and 3E). Treatment with glucagon had no significant effect on ACC Ser79 phosphorylation (Figures 3B and 3F). The transient rapid and transient decrease in GYS activity, which correlated with AMPK or PKA together with CK1 (Figure 2E). Interestingly, glucagon treatment, however, induced a sharp contrast with glucagon treatment of rat hepatocytes, where transient changes in GYS activity and GYS2 Ser7 phosphorylation in contrast with glucagon treatment of WT mouse hepatocytes, where transient changes in GYS activity and GYS2 Ser7 phosphorylation in hepatocytes from WT mice incubated with AICA riboside, A769662 or glucagon, GYS activity decreased rapidly, reaching ~40%, 50% and 90% inactivation after 15 min of incubation respectively (Figure 4F). Thus GYS inactivation by the various treatments in rat and WT mouse hepatocytes was greater than that seen in vitro following AMPK- and PKA-induced phosphorylation of recombinant GYS2 (Figures 2C and 2D), but was similar to the extent of recombinant GYS inactivation seen after phosphorylation by AMPK or PKA together with CK1 (Figure 2E). Interestingly, in contrast with glucagon treatment of rat hepatocytes, where transient changes in GYS activity and GYS2 Ser7 phosphorylation were observed (Figures 3B and 3F), GYS inactivation in response to glucagon treatment of WT mouse hepatocytes was maintained over the 30 min incubation period (Figure 4F). In addition, in WT mouse hepatocytes, incubation with both glucagon and AICA riboside increased GP Ser14 phosphorylation (Figure 4C), whereas incubation with A769662 or glucagon had no significant effect (Figures 4A and 4E). In hepatocytes from WT mice, we also confirmed that treatment with AICA riboside or A769662 increased ACC Ser79 phosphorylation, whereas incubation with glucagon had no effect on phosphorylation at this target site (Figures 4B and 4E). In hepatocytes from WT mice incubated with AICA riboside, A769662 or glucagon, GYS activity decreased rapidly, reaching ~40%, 50% and 90% inactivation after 15 min of incubation respectively (Figure 4F). Thus GYS inactivation by the various treatments in rat and WT mouse hepatocytes was greater than that seen in vitro following AMPK- and PKA-induced phosphorylation of recombinant GYS2 (Figures 2C and 2D), but was similar to the extent of recombinant GYS inactivation seen after phosphorylation by AMPK or PKA together with CK1 (Figure 2E). Interestingly, in contrast with glucagon treatment of rat hepatocytes, where transient changes in GYS activity and GYS2 Ser7 phosphorylation were observed (Figures 3B and 3F), GYS inactivation in response to glucagon treatment of WT mouse hepatocytes was maintained over the 30 min incubation period (Figure 4F). In addition, in WT mouse hepatocytes, incubation with both glucagon and AICA riboside increased GP Ser14 phosphorylation (Figure 4C), although the increase with glucagon was not transient, as observed in rat hepatocytes (compare Figures 4C and 3G).

In hepatocyte extracts from AMPK<sub>α1/α2LS</sub>−/− mice, basal GYS activity was almost 2-fold higher (831 ± 72 m-units/g of cells) than in extracts from WT mice (456 ± 28 m-units/g of cells) (Figures 4F and 4G). In hepatocyte extracts from AMPK<sub>α1/α2LS</sub>−/− mice incubated with AICA riboside or A769662, no signals for AMPK Thr172 phosphorylation, ACC Ser79 phosphorylation or total α1/α2 AMPK by immunoblotting were detected (Figure 4E). In addition, AMPK activity in hepatocytes from α1/α2 LS-KO (knockout) mice was substantially reduced and, compared with hepatocytes from WT mice, was not increased by AICAR treatment (Supplementary Figure S1 at http://www.BiochemJ.org/bj/443/bj4430193add.htm). Glucagon treatment of hepatocytes from AMPK<sub>α1/α2LS</sub>−/− mice induced a rapid time-dependent decrease in GYS activity to an extent similar to that observed in hepatocytes from WT mice (Figures 4F and 4G). In hepatocytes from AMPK<sub>α1/α2LS</sub>−/−

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### Table 1 Effects of phosphorylation by AMPK and PKA on the kinetic properties of recombinant GYS1 and GYS2

Summary of the values of K<sub>m</sub> for UDP-Glc and V<sub>max</sub> measured with or without 10 mM Glc-6-P and K<sub>a</sub> for Glc-6-P after curve-fitting of the data shown in Figure 2. Results are means ± S.E.M. for three independent experiments. *P < 0.05 compared with the control values (paired two-sided Student's t test).

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<td><strong>K&lt;sub&gt;m&lt;/sub&gt; (mM)</strong></td>
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<tr>
<td>Glc-6-P Control</td>
<td>0.32 ± 0.04</td>
<td>0.39 ± 0.05</td>
<td>8.62 ± 0.28</td>
<td>10.07 ± 0.34</td>
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<tr>
<td>AMPK</td>
<td>0.15 ± 0.02</td>
<td>0.15 ± 0.02</td>
<td>8.04 ± 0.26</td>
<td>10.76 ± 0.32</td>
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<tr>
<td>PKA</td>
<td>0.13 ± 0.03</td>
<td>0.18 ± 0.02</td>
<td>7.77 ± 0.34</td>
<td>12.10 ± 0.26</td>
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<tr>
<td><strong>V&lt;sub&gt;max&lt;/sub&gt; (μmol/min per mg of protein)</strong></td>
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<tr>
<td>Glc-6-P Control</td>
<td>8.62 ± 0.28</td>
<td>10.07 ± 0.34</td>
<td>8.54 ± 0.56</td>
<td>10.69 ± 0.47</td>
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<tr>
<td>AMPK</td>
<td>8.04 ± 0.26</td>
<td>10.76 ± 0.32</td>
<td>7.77 ± 0.34</td>
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| **K<sub>a</sub> for Glc-6-P (mM)** |        |        |
| GYS1 | 0.07 ± 0.03 | 0.04 ± 0.01 |
| GYS2 | 0.13 ± 0.03* | 0.12 ± 0.03* |

Effects of pharmacological AMPK activators on GYS inactivation in freshly isolated hepatocytes prepared from liver-specific AMPK α1/α2-deficient mice

As observed in rat hepatocytes, AMPK Thr172 phosphorylation increased in WT mouse hepatocytes incubated with AICA riboside, whereas incubation with A769662 or glucagon had no significant effect (Figures 4A and 4E). In hepatocytes from WT mice, we also confirmed that treatment with AICA riboside or A769662 increased ACC Ser79 phosphorylation, whereas incubation with glucagon had no effect on phosphorylation at this target site (Figures 4B and 4E). In hepatocytes from WT mice incubated with AICA riboside, A769662 or glucagon, GYS activity decreased rapidly, reaching ~40%, 50% and 90% inactivation after 15 min of incubation respectively (Figure 4F). Thus GYS inactivation by the various treatments in rat and WT mouse hepatocytes was greater than that seen in vitro following AMPK- and PKA-induced phosphorylation of recombinant GYS2 (Figures 2C and 2D), but was similar to the extent of recombinant GYS inactivation seen after phosphorylation by AMPK or PKA together with CK1 (Figure 2E). Interestingly, in contrast with glucagon treatment of rat hepatocytes, where transient changes in GYS activity and GYS2 Ser7 phosphorylation were observed (Figures 3B and 3F), GYS inactivation in response to glucagon treatment of WT mouse hepatocytes was maintained over the 30 min incubation period (Figure 4F). In addition, in WT mouse hepatocytes, incubation with both glucagon and AICA riboside increased GP Ser14 phosphorylation (Figure 4C), although the increase with glucagon was not transient, as observed in rat hepatocytes (compare Figures 4C and 3G).
AMPK inactivation of liver glycogen synthase

Figure 3  Time course of the effects of AICA riboside, A769662 and glucagon on AMPK activity, AMPK Thr172, ACC Ser79 and GYS2 Ser7 phosphorylation in incubated rat hepatocytes

Freshly isolated rat hepatocytes were incubated for 30 min with 30 μM A769662, 1 mM AICA riboside or 10−7 M glucagon compared with vehicle control (CTL). At the indicated times, the cells were freeze-stopped and extracts were prepared for the measurement of AMPK (A), GYS (B) and GP (C) activity and for immunoblotting AMPK Thr172 (D), ACC Ser79 (E), GYS2 Ser7 (F) and GP Ser14 (G) phosphorylation. In (D and E) the histograms show the quantification of immunoblots by Odyssey TM imaging after ratios of the signals obtained with the anti-phospho compared with anti-(total protein) antibodies had been calculated. In (F and G) pGYS2 Ser7 and pGP Ser14 phosphorylation were expressed as fold increases after immunoblot detection by chemiluminescence. In (H) a representative immunoblot for pGYS2 Ser7 phosphorylation compared with eEF2 as a loading control is shown. Results are means ± S.E.M. for four independent experiments. * P < 0.05 compared with the control values (paired two-sided Student’s t test).

mice, GYS inactivation by AICA riboside was considerably reduced compared with hepatocytes from WT mice, whereas in response to A769662, GYS2 was inactivated by approximately 60% in spite of the loss of the AMPK catalytic subunits (Figures 4F and 4G). In hepatocytes from both WT and AMPKα1α2LS−/− mice incubated with glucagon or AICA riboside, GP Ser14 phosphorylation increased, but did not change in response to A769662 (Figures 4C and 4D). Unfortunately, immunoblotting of hepatocyte extracts from AMPKα1α2LS−/− mice for GYS2 Ser7 phosphorylation was inconclusive because of
Figure 4  Time-course of the effects of AICA riboside, A769662 and glucagon on AMPK Thr172, ACC Ser79 phosphorylation and GYS activity in incubations of hepatocytes from WT and AMPKα1α2LS−/− mice

Freshly isolated hepatocytes from WT and AMPKα1α2LS−/− mice were incubated for 30 min with 30 μM A769662, 1 mM AICA riboside or 10−7 M glucagon compared with vehicle control (CTL). At the indicated times, the cells were freeze-stopped and extracts were prepared for immunoblotting AMPK Thr172 (A), ACC Ser79 (B) and GP Ser14 (C) phosphorylation from WT hepatocyte incubations and GP Ser14 (D) phosphorylation from AMPKα1α2LS−/− hepatocyte incubations, as described in the legend to Figure 3. (E) A representative immunoblot of AMPK Thr172, ACC Ser79 and total AMPK (α1/α2) with pyruvate carboxylase revealed by the use of streptavidin-coupled horseradish peroxidase as a loading control. GYS activities in extracts from WT (F) and AMPKα1α2LS−/− (G) hepatocyte incubations are also shown. Results are means ± S.E.M. for three (F and G) or four (A–D) independent experiments. * P < 0.05 compared with the control values (paired two-sided Student’s t test).
limited amounts of material available from only three separate experiments.

**DISCUSSION**

In the present study we show that both AMPK and PKA phosphorylate recombinant GYS2 to the same maximal stoichiometry and at a single site identified as Ser7. GYS2 phosphorylation by AMPK or PKA led to enzyme inactivation by decreasing the affinity of the enzyme for both UDP-Glc (in the absence of Glc-6-P) and Glc-6-P (at low UDP-Glc concentrations) without affecting $V_{\text{max}}$. Very similar effects of AMPK- and PKA-induced phosphorylation on the kinetic properties of GYS1 were observed. In skeletal muscle, AMPK activation via AICA riboside treatment results in GYS inactivation, which can largely be overcome by the rise in Glc-6-P due to the stimulation of glucose transport [6], explaining why glycogen synthesis can increase as a result of acute or repeated AICA riboside injection in rodents [42–44]. Phosphorylation of GYS1 at site 2 is a priming event for phosphorylation at site 2a by CK1, which inactivates the enzyme to a greater extent than seen after phosphorylation by PKA alone [10]. For GYS2, we observed that phosphorylation by both AMPK and CK1 led to inactivation, even when assayed with high concentrations of UDP-Glc (Figure 2E). This explains why GYS activity was substantially decreased in hepatocytes subjected to the various treatments, presumably due to phosphorylation at both sites 2 and 2a.

In freshly isolated rat hepatocytes, AMPK activation was seen on incubation with both AICA riboside and A769622, as reflected by increases in both AMPK Thr172 and ACC Ser79 phosphorylation (Figures 3D and 3E). However, A769622 treatment did not increase AMPK activity as measured using a peptide-based assay (Figure 3A), which is in contrast with previous reports [45–47]. A762662 can both allosterically stimulate AMPK and promote AMPK α-subunit Thr172 phosphorylation [45,48], but the allosteric effect is lost during AMPK assay of poly(ethylene glycol) fractions from cell extracts. However the increase in downstream ACC phosphorylation is testimony to allosteric stimulation of AMPK by A769622 treatment in the present study (Figure 3E). Although A769622 caused a very modest, but significant, increase in Thr172 phosphorylation, our AMPK assay might not have been sensitive enough to detect this small increase. There are other reports in the literature showing that, in hepatocytes incubated with A769622, ACC phosphorylation was increased without a rise in AMPK α-subunit Thr172 phosphorylation [24,48]. The differences between these results and those previously reported [45–47] might be due to the use of different cell models (hepatocytes compared with HEK-293, CCL13 cells and mouse embryonic fibroblasts), A769622 concentrations or duration of treatment.

AMPK activation by treatment with A769622 or AICA riboside induced persistent GYS2 inactivation and Ser7 phosphorylation. Incubation with glucagon induced transient GYS2 inactivation and Ser7 phosphorylation due to activation of GYS phosphatase in the presence of glucose following inactivation of GP. This inverse relationship between the active forms of GP and GYS in liver was elegantly demonstrated many years ago following glucose administration to mice [49]. The fact that pharmacological AMPK activators induced a persistent inactivation of GYS2 indicates that AMPK might rapidly negatively regulate the activity of PP1c complexes involved in the control of glycogen metabolism. It is noteworthy that, in the long term, AMPK phosphorylates the R5/PTG (protein-targeting glycogen) subunit of the R5/PTG–PP1c complex to accelerate its degradation via the malin–laforin complex [50].

Treatment of freshly isolated hepatocytes from WT mice with A769662, AICA riboside or glucagon led to GYS2 inactivation. However, in mouse hepatocytes incubated with glucagon, GYS inactivation was rapid, but not transient, and was sustained during the course of incubation, presumably because PP1 regulation is different in mouse and rat. In hepatocytes from AMPK$_{1a,2LS-/-}$ mice, GYS activities were increased ∼2-fold compared with activities in WT mice, suggesting that AMPK may have a negative effect on GYS2 gene transcription or translation. Indeed increased levels in GYS2 protein in hepatocyte extracts from AMPK$_{1a,2LS-/-}$ mice were seen by immunoblotting (results not shown). It is noteworthy that in peroxisome-deficient hepatocytes where AMPK became activated, GYS expression decreased along with glycogen synthesis [51]. GYS inactivation by AICA riboside treatment was severely blunted in hepatocytes from AMPK$_{1a,2LS-/-}$ mice (Figure 4G) compared with WT mice (Figure 4F). However, following treatment with A769622, there was still 50–60% inactivation of GYS2 in hepatocytes from AMPK$_{1a,2LS-/-}$ mice (Figure 4G). It is now becoming clear, though, that A769662 has AMPK-independent effects, such as the inhibition of glucose production in hepatocytes from β1-KO [46] and α1 or 2 LS-KO mice [24].

On the basis of our findings, we propose that AMPK activation in hepatocytes leads to GYS inactivation mediated by Ser7 phosphorylation. AMPK activation in liver during exercise would thus be expected to decrease glycogen synthesis and favour glycolysis to maintain ATP levels and reduce gluconeogenesis. AMPK has been shown to bind glycogen via the glycogen-binding domain of its β-subunits [52]. Moreover, branched oligosaccharides and limit dextrins, characteristic of highly degraded glycogen, inhibit AMPK activity [53] such that GYS2 would be active to replenish glycogen reserves following liver glycogen depletion during exercise. In addition, AMPK would bind glycogen via its β-subunit glycogen-binding domain to target it to GYS2 for phosphorylation by AMPK near the appropriate conditions. Transgenic mouse models with knock-in mutations (GYS2 S7E and S7E/S10E, and mutations to abolish allosteric stimulation by Glc-6-P) would be useful to further delineate the role of control of GYS2 by AMPK in liver glycogen metabolism.

**AUTHOR CONTRIBUTION**

Laurent Bultot carried out most of the experimental work in the Brussels and Dundee laboratories. Bruno Guigas and Liliane Maisin performed mouse and rat hepatocyte incubations. Monique Beullens provided reagents and advice for appropriate conditions. Transgenic mouse models with knock-in mutations (GYS2 S7E and S7E/S10E, and mutations to abolish allosteric stimulation by Glc-6-P) would be useful to further delineate the role of control of GYS2 by AMPK in liver glycogen metabolism.

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SUPPLEMENTARY ONLINE DATA

AMP-activated protein kinase phosphorylates and inactivates liver glycogen synthase

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Figure S1 Time course of the effects of AICA riboside, A769662 and glucagon on AMPK activity in incubations of hepatocytes from WT and AMPKα1α2LS−/− mice

Freshly isolated hepatocytes from WT and AMPKα1α2LS−/− mice were incubated for 30 min with 30 μM A769662, 1 mM AICA riboside or 10−7 M glucagon compared with vehicle control (CTL). At the indicated times, the cells were freeze-stopped and extracts were prepared for the measurement of AMPK activity. Results are means ± S.E.M. for three independent experiments. Compared with hepatocytes from WT mice at zero time, AMPK activity in hepatocytes from AMPKα1α2LS−/− mice was significantly reduced [6.65 ± 1.28 compared with 1.82 ± 0.17 nmol/min per g of cells, P < 0.05 (paired two-sided Student's t test)].

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