

## Characterization of AMP-activated protein kinase $\gamma$ -subunit isoforms and their role in AMP binding

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The AMP-activated protein kinase (AMPK) cascade plays an important role in the regulation of energy homeostasis within the cell. AMPK is a heterotrimer composed of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ). We have isolated and characterized two isoforms of the  $\gamma$  subunit, termed  $\gamma 2$  and  $\gamma 3$ . Both  $\gamma 2$  (569 amino acids) and  $\gamma 3$  (492 amino acids) have a long N-terminal domain which is not present in the previously characterized isoform,  $\gamma 1$ . As with  $\gamma 1$ , mRNA encoding  $\gamma 2$  is widely expressed in human tissues, whereas significant expression of  $\gamma 3$  mRNA was only detected in skeletal muscle. Using isoform-specific antibodies, we determined the AMPK activity associated with the different  $\gamma$  isoforms in a number of rat tissues. In most tissues examined more than 80% of total AMPK activity was associated with the  $\gamma 1$  isoform, with the remaining activity being accounted for mainly by the  $\gamma 2$  isoform. Exceptions to this were

testis and, more notably, brain where all three isoforms contributed approximately equally to activity. There was no evidence for any selective association between the  $\alpha 1$  and  $\alpha 2$  isoforms and the various  $\gamma$  isoforms. However, the AMP-dependence of the kinase complex is markedly affected by the identity of the  $\gamma$  isoform present, with  $\gamma 2$ -containing complexes having the greatest AMP-dependence,  $\gamma 3$  the lowest, and  $\gamma 1$  having an intermediate effect. Labelling studies, using the reactive AMP analogue 8-azido-<sup>32</sup>P]AMP, indicate that the  $\gamma$  subunit may participate directly in the binding of AMP within the complex.

**Key words:** allosteric activation, cell signalling, metabolic regulation.

### INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) is activated by stresses which deplete ATP, leading to a rise in the AMP:ATP ratio within the cell [1–3]. AMPK is activated allosterically by AMP and this effect is antagonized by ATP. In addition, AMPK is activated by phosphorylation catalysed by an upstream kinase, the AMP-activated protein kinase kinase (AMPKK), which is itself activated by AMP [4]. AMP has also been shown to activate AMPK by making it a better substrate for AMPKK and a worse substrate for protein phosphatases [5]. These effects make AMPK acutely sensitive to changes in the AMP:ATP ratio [3,6] and have led to the suggestion that AMPK may act as a cellular fuel gauge [1]. Once activated, AMPK switches off a number of ATP-consuming pathways, including fatty acid and cholesterol synthesis [7], thereby conserving the immediate energy expenditure of the cell. Another consequence of AMPK activation that is beginning to emerge is an increase in the supply of fuel, or ATP, available to the cell. AMPK increases the rate of fatty acid oxidation by inactivating acetyl-CoA carboxylase and causing a decrease in malonyl-CoA [8–11]. Recently, evidence has accumulated indicating that AMPK may also increase the rate of glucose oxidation in muscle by increasing the rate of glucose transport [12,13]. Taken together, these findings suggest

that the AMPK cascade plays an important role in restoring the energy balance of the cell in response to a decrease in ATP during periods of cellular stress.

AMPK is composed of one catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ) which associate to form a heterotrimer [14,15]. Expression studies in mammalian cells have shown that all three subunits are required to yield significant kinase activity [16]. Isoforms of the  $\alpha$  subunits [17] and  $\beta$  subunits [18] have been identified. Although the precise physiological relevance of these isoforms remains unknown, the expression pattern of the isoforms varies dramatically between tissues [17,18]. It is possible that the isoforms play a role in providing tissue-specific function and/or regulation of the AMPK complex. For instance, the  $\alpha 2$  and  $\beta 2$  isoforms are highly expressed in skeletal and cardiac muscle [18,19]; a recent report indicated that  $\alpha 2$ -containing complexes, but not  $\alpha 1$ -containing complexes, contributed to the activation of AMPK in muscle following electrical stimulation [10]. In addition, we previously reported differences in the subcellular localization and AMP-dependence of  $\alpha 1$ - and  $\alpha 2$ -containing complexes [20].

Homologues of all three AMPK subunits are present in the sucrose non-fermenting (SNF1) complex from the yeast, *Saccharomyces cerevisiae*. This complex, which is essential for the expression of glucose-repressed genes [21], is also thought to be a

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; ASC, association with SNF1 complex; BLAST, Basic Local Alignment Search Tool; CBS, cystathionine  $\beta$ -synthase; DTT, dithiothreitol; EST, expressed sequence tag; FSBA, *p*-fluorosulphonylbenzoyl adenosine; KIS, kinase interacting sequence.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AJ249976 (AMPK- $\gamma 2$ ) and AJ249977 (AMPK- $\gamma 3$ ).

heterotrimer composed of a catalytic subunit (Snf1p; 72 kDa), a regulatory subunit (Snf4p; 36 kDa) and a third subunit (one member of the Sip1p/Sip2p/Gal83p family) [2]. We refer to the active complex as SNF1, and the individual subunits as Snf1p, Snf4p, etc. The Sip1p/Sip2p/Gal83p proteins are highly related and contain within them sequences termed the KIS (kinase interacting sequence) and ASC (association with SNF1 complex) domains, which bind to Snf1p and Snf4p respectively [22]. The AMPK  $\beta$ -subunit isoforms contain sequences highly related to the KIS and ASC domains found in the Sip1p/Sip2p/Gal83p proteins [18], and it seems likely that  $\alpha$  and  $\gamma$  bind to the  $\beta$  subunit via interaction with these sequences. Binding of the  $\beta$  and  $\gamma$  subunits within the heterotrimer appears to stabilize the  $\alpha$  subunit, increasing its half-life approximately twofold [23]. Formation of the heterotrimeric complex may therefore be important not only for maximal kinase activity but also for stabilization of the enzyme.

We report here the isolation of cDNA clones encoding two new  $\gamma$ -subunit isoforms. We have compared the tissue distribution of these isoforms with the previously identified  $\gamma$ 1 isoform. We also investigated the binding of AMP within the AMPK complex and provide evidence that the  $\gamma$  subunit participates directly in this process. Our results provide further evidence that AMPK exists as an heterogeneous pool of iso-enzymes within the cell that may respond differently to changes within the AMP:ATP ratio.

## EXPERIMENTAL

### Identification of the $\gamma$ 2 isoform

The amino acid sequence of human AMPK $\gamma$ 1 [24] was used to search the EST (expressed sequence tag) database using the BLAST (Basic Local Alignment Search Tool) program [25]. The sequence of one of the clones identified from this search (accession number AA434293) was used in a subsequent search of the database. Four clones (accession numbers R32671, H64260, AA059138 and AA131886) were obtained from the IMAGE consortium. Plasmid DNA from the clones was prepared and analysed by digestion with various restriction enzymes in order to identify the clone harbouring the longest insert (AA059138) which was sequenced in its entirety.

### Two-hybrid library screening-identification of the $\gamma$ 3 isoform

A human skeletal muscle library in pGAD10 (ClonTech) was screened using the C-terminal 201 amino acids of rat  $\beta$ 1 [15] fused to the DNA-binding domain of Gal4, using a method described previously for screening of this library with rat AMPK $\alpha$ 2 [18]. Briefly, the C-terminal 201 amino acids of  $\beta$ 1 were amplified and cloned into the *Sal*I and *Nco*I sites of pYTH9. The resulting plasmid, pYTH9- $\beta$ 1, was linearized by digestion with *Xba*I and integrated into Y190 strain yeast. Approx.  $1.7 \times 10^6$  colonies were screened and positive clones were identified by the appearance of blue colonies following incubation with 5-bromo-4-chloroindol-3-yl  $\beta$ -D-galactopyranoside. cDNA inserts in pGAD10 were amplified from yeast extracts and the products analysed by electrophoresis either before or after digestion with either *Rsa*I or *Alu*I. Clones were grouped according to their restriction digest pattern and an individual clone from each group was isolated from the original yeast colonies and transformed into *Escherichia coli*. The inserts were sequenced by the dideoxynucleotide chain-termination method using Sequenase (version 2.0 from Amersham Pharmacia Biotech) in accordance with the manufacturer's instructions.

### Interactions using the two-hybrid system

Vectors (ClonTech) expressing the GAL4 DNA binding domain (pGBT9) and the GAL4 activation domain (pGAD424) were used to prepare fusions with the various AMPK-subunit isoforms. The entire coding sequence of  $\gamma$ 2 was amplified from clone AA059138 (forward primer: GAATTCGGCGGCCG-CGCGAGTTCCC; reverse primer: CTGCAGCCGGGCTC-CG TTTCTGTCTCCTTTT), digested with *Eco*RI and *Pst*I and cloned into pGBT9. pGAD10- $\gamma$ 3 (isolated from the two-hybrid library screen) was digested with *Eco*RI and the insert, containing the entire coding sequence of  $\gamma$ 3, was cloned into pGBT9. Vectors expressing fusions of the  $\alpha$  and  $\beta$  subunits, and of the  $\gamma$ 1 isoform have been described previously [15,18]. Yeast (strain SFY526 harbouring a GAL1-*lacZ* reporter gene) were transformed with various combinations of the vectors and grown on selective media. To test for interactions, several colonies from each transformation were patched onto selective media and grown for 2 days at 30 °C. Colonies were transferred to nitrocellulose filters and cells were permeabilized in liquid nitrogen. The filters were incubated in the presence of 5-bromo-4-chloroindol-3-yl  $\beta$ -D-galactopyranoside, and the time taken for any blue colouration to appear was noted.

### In vitro translations

The entire coding sequences of human  $\gamma$ 1,  $\gamma$ 2 and  $\gamma$ 3 cDNAs were amplified and cloned into pCDNA3 (Invitrogen). RNA transcripts were synthesized using T7 polymerase, and translated in reticulocyte lysates using a coupled transcription/translation system (TNT system; Promega) in the presence of [<sup>35</sup>S]-methionine. Total labelled products of translation were analysed by SDS/PAGE and fluorography.

### Northern-blot analysis

A human multiple tissue Northern blot (ClonTech) was incubated with either a random primed human AMPK $\gamma$ 1, AMPK $\gamma$ 2 or AMPK $\gamma$ 3 cDNA probe. Following hybridization, the blots were washed with  $2 \times$  SSC (300 mM NaCl/30 mM sodium citrate, pH 7.0), 0.5% SDS at room temperature for 1 h, followed by a second wash at 60 °C for  $2 \times 1$  h, and exposed to autoradiograph film for 1–2 days at  $-70$  °C.

### Cell culture

H4IIE cells were grown in Dulbecco's modified Eagle's medium, supplemented with 2.5% foetal calf serum, 2.5% newborn calf serum, 100 units/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were maintained at 37 °C in 95% air/5% CO<sub>2</sub>.

### Preparation of various tissue extracts for AMPK assay

Tissues were dissected from a male rat (body weight approx. 250 g) and placed in ice-cold homogenization buffer [50 mM Tris/HCl (pH 8.4), 50 mM NaF, 5 mM sodium pyrophosphate, 250 mM mannitol, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM benzamide, 0.1 mM PMSF, 1  $\mu$ g/ml soybean trypsin inhibitor]. Tissues were then washed to remove blood, trimmed, and homogenized in 20 ml of buffer using a Polytron homogenizer (Merck), followed by 5 passes in a Dounce homogenizer (Merck). The homogenates were then centrifuged



trifluoroethane to remove the perchloric acid. Complete conversion of ATP into AMP was demonstrated by HPLC analysis on a Whatman Partisil-10 SAX column. The recovery of azido-AMP from azido-ATP was 92%. For the affinity labelling experiment, 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{AMP}$  (2.4 mM; 107 d.p.m./nM) was incubated with AMPK (10.5 mg/ml; purified as far as the gel-filtration step) in 50 mM Tris/HCl, pH 7.4 (4 °C), 10% (w/v) glycerol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 0.02% (w/v) Brij 35, for 5 min at room temperature, in the presence or absence of 20  $\mu\text{M}$  and 100  $\mu\text{M}$  AMP. The mixture was then exposed on ice to UV light in a UV Stratalinker 2400 (Stratagene), using the AutoCrosslink program. Samples were then analysed by SDS/PAGE, the gel dried and subjected to autoradiography.

## RESULTS

### Isolation of cDNA clones encoding $\gamma 2$ and $\gamma 3$

A search of the EST database with the amino acid sequence of human  $\gamma 1$  identified a number of clones with sequences, which, although highly related to  $\gamma 1$ , clearly represented a different isoform. The sequence of one of these clones (with accession number AA434293) was used for a subsequent search of the database. Four clones, with sequences which matched that of AA434293, were obtained from the IMAGE consortium through the UK Human Genome Mapping Resource Centre, Cambridge. Preliminary analysis of the inserts from these clones revealed that the clone containing the longest insert corresponded to AA059138. This clone was sequenced in its entirety and the nucleotide and predicted amino acid sequences are shown in Figure 1. A partial sequence, which was also identified by searching the EST database, corresponding to residues 318–569 of the sequence reported here has been published previously [30]. The full-length  $\gamma 2$  cDNA encodes a polypeptide of 569 amino acids with a calculated molecular mass of 63 kDa.

Screening of a skeletal muscle two-hybrid library with the C-terminal 201 amino acids of  $\beta 1$ , identified a number of interacting clones. Sequence analysis revealed that six clones encoded  $\gamma 1$  and one clone encoded a partial sequence corresponding to  $\alpha 2$ . None of the clones we identified encoded  $\gamma 2$ . Two clones, however, encoded a protein which shares considerable sequence identity with the  $\gamma 1$  and  $\gamma 2$  isoforms. We designated these clones  $\gamma 3$  and the nucleotide and predicted amino acid sequences are shown in Figure 2. The  $\gamma 3$  cDNA encodes a polypeptide of 492 amino acids with a calculated molecular mass of 55 kDa.

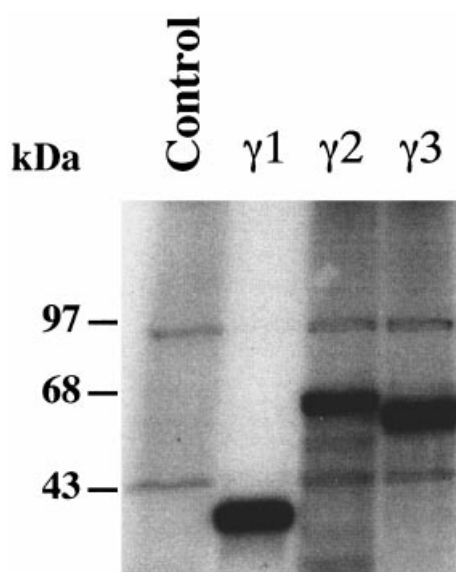
The predicted sizes of the  $\gamma 2$  and  $\gamma 3$  polypeptides are significantly larger than that of the  $\gamma 1$  isoform (331 amino acids, molecular mass 37 kDa). RNA encoding each of the  $\gamma$  isoforms was synthesized *in vitro*, translated using a rabbit reticulocyte lysate system, and the products analysed by SDS/PAGE and fluorography (Figure 3). In each case a major labelled polypeptide corresponding to the predicted mass of the appropriate isoform was detected. Minor labelled products of approx. 90 kDa and 43 kDa were also detected in the  $\gamma 2$  and  $\gamma 3$  translations, but these were also present in a control reaction performed in the absence of RNA. Alignment of the amino acid sequences of the  $\gamma$  isoforms reveals that, compared to  $\gamma 1$ , both  $\gamma 2$  and  $\gamma 3$  have long N-terminal extensions (approx. 230 amino acids in  $\gamma 2$  and 150 amino acids in  $\gamma 3$ ; results not shown). The N-terminal regions of  $\gamma 2$  and  $\gamma 3$  show no obvious sequence identity with one another or with any other sequences in the database. In contrast, the C-terminal ~300 amino acids of the  $\gamma 2$  and  $\gamma 3$  isoforms share significant amino acid sequence identity with  $\gamma 1$  ( $\gamma 1$  versus  $\gamma 2$ , 77% identity;  $\gamma 1$  versus  $\gamma 3$ , 63% identity;  $\gamma 2$  versus  $\gamma 3$ , 63%

1	acttggtctggggctggccacatgagccggcgctggagcagcactgcccaggaccctc	60
	<b>M E P G L E H A L R R T P</b>	
14	tcttgaggacgacctgggggtctgagcatcaagagatgagcttctagagcaagaaac	120
	<b>S W S S L G G S E H Q E M S F L E Q E N</b>	
34	agcagctcatggccatcaccagctgtgaccagcagctcagaaagaatccgtgggaacg	180
	<b>S S S W P S P A V T S S E I S P N D S L</b>	
54	aggggcaaacgcttgagatggagcagcagaagtcgggtgaggaggagccaccaggt	240
	<b>R A K A L R W T R Q K S V E E G E P P G</b>	
74	cagggggaaggtcccggctcaggccagctgctgagctccacgggctggaggccattc	300
	<b>Q G E G P R S R P A A E S T G L E A T F</b>	
94	cccaagaccacacccttggtcaagctgactcctggcgctggcactccaccaacagg	360
	<b>P K T T P L A Q A D P A G V G T P P T G</b>	
114	tggaactgctccctctgactgtacagcctcagctcagctccagctccagacagatg	420
	<b>W D C L P S D C T A S A A G S S T D D V</b>	
134	gagctggccagaggtccaccagcagagcctgggagtgtagctagaagcctgct	480
	<b>E L A T E F P A T E A W E C E L E G L L</b>	
154	gaagagagcctgcccctgctgctgctcccagcagcccccatttccaagctggctgg	540
	<b>E E R P A L C L S P Q A P F P K L G W D</b>	
174	gacgaactgcccgaacccggcagcagctacatcgcttcatcagcagcagcagctc	600
	<b>D E L R K P G A Q I Y M R F I E E H T C</b>	
194	tacgatgcatggcaactagctccaagctagctcattctcgaccaccatgctggagat	660
	<b>Y D A M A T S S K L V I F D T M L E I K</b>	
214	aagcctcttctgctctggtggccaagctggtggggcagccctctatgggacagcaag	720
	<b>K A F F A L V A N G V R A A P L W D S K</b>	
234	aagcagacttctggtgggctgctgacctgactgactcctgctgctgctgctgctac	780
	<b>K Q S F V G M L T I T D F I L V L H R Y</b>	
254	tacaggtcccctggtccagatctatgagattgaacaacataagattgagactggagg	840
	<b>Y R S P L V Q I Y E I E Q H K I E T W R</b>	
274	gagatctacctgcaagctgcttcaagcctctgctcctctcctaatgatagcctg	900
	<b>E I Y L Q G C F K P L V S I S P N D S L</b>	
294	tttgaagctgtctacaccctcatcaagaacggatccatcctgctgcttcttggaccg	960
	<b>F E A V Y T L I K N R I H R L P V L D P</b>	
314	gtgtcagcgaactgactccacatcctcacacacaagcctgctcagcttctcctgacatc	1020
	<b>V S G N V L H I L T H K R L L K F L H I</b>	
334	tttggttccctgctgcccggcctcctctcctcctcacttccaagatttgggctc	1080
	<b>F G S L L P R P S L Y R T I Q D L G I</b>	
354	ggcacatcccgagactggctggtgctgagcagcagccaccctcctgactgactggac	1140
	<b>G T F R D L A V V L E T A P I L T A L D</b>	
374	atcttvtgagcagcggctgtgctgctgactgctgctgctgctgctgctgctgctg	1200
	<b>I F V D R R V S A L P V V N E C G Q V T</b>	
394	ggcctctattccccttctgctgctgctgctgctgctgctgctgctgctgctgctg	1260
	<b>G L Y S R F D V I H L A A Q Q T Y N H L</b>	
414	gacatgagtgaggagaagcctgaggaagagcactatgcttctgaggagctcctctcc	1320
	<b>D M S V G E A L R K R T L C L E G V L S</b>	
434	tgcccacccacagagacttggggagtgatgacagcagcctcctgggagcaggtacac	1380
	<b>C Q P H E S L G E V I D R I A R E Q V H</b>	
454	aggctggtgctagtgagcagcagcagcagcagcagcagcagcagcagcagcagcagc	1440
	<b>R L V L V D E T Q H L L G V V S L S D I</b>	
474	cttcaggcactggtgctcagcctgagcctgagcctcctcctcctcctcctcctcctc	1500
	<b>L Q A L V L S P A G I D P S G P E K I ***</b>	
	gtcctcaatcccaagccctgacacactggaagcgaatgaaggaactggagaactcag	1560
	ccttcatctccccaccccccttctgctgctcagctatgattcaggtctctcagggcc	1620
	tcccaaatgcccctgcccaccagagctcccagaagcctcggcatgccagctgacccat	1680
	gggatgataaataaggaagacagctgagctcagctcagctcagctcagctcagctcag	1740
	ctaggtatccccagggccctctgctcctcctcctcctcctcctcctcctcctcctcct	1800
	ggtcggatggccccagtggtttagctcagggcttctggaatcctcctggttctctgggtc	1860
	tatgcttcaactcagctcctggggagtcccagcaagctgcccactgcccctcctactct	1920
	ccaggggttggtcattcaaggtcctgaaatgctgcaattcagggggccaccatggagc	1980
	agccttattatagaactgcccctggtgaggtggggagctcctccatctcttctgcca	2040
	gaaaaactccttagctctcagctgagccatgcttcttagctccagggatggatggcct	2100
	gtatctggaccctgagatgagcaattgagaaacacaaaggaacacacacacacacacac	2160
	cttagattttatggttccactcaaaaagctcagctcatttgcaactgaaacttgggcaag	2220
	agccttctgcttctcaaatcaaaagactagaagggaaatggataaaaatcacaagtgcg	2280
	tttctcttgc 2340	

**Figure 2** Nucleotide sequence and predicted amino acid sequence of human AMPK $\gamma 3$

Nucleotides are numbered on the right and amino acids on the left. The putative initiation codon and stop codon are shown in **bold**.

identity). An alignment of the C-terminal regions of  $\gamma 2$  and  $\gamma 3$  with the entire coding sequence of  $\gamma 1$  isoforms is shown in Figure 4.



**Figure 3** *In vitro* translation of human AMPK $\gamma$  isoforms

[<sup>35</sup>S]Methionine-labelled  $\gamma$  isoforms were translated *in vitro* in rabbit reticulocyte lysates. Protein products were resolved by SDS/PAGE and visualized by fluorography. A control reaction in the absence of added RNA was included. The migration of molecular mass markers is shown on the left.

#### Northern-blot analysis of the $\gamma$ isoforms

Polyadenylated-rich RNA isolated from a number of human tissues was probed with cDNA encoding  $\gamma 1$ ,  $\gamma 2$  or  $\gamma 3$ . Results are shown in Figure 5. Human  $\gamma 1$  mRNA was detected as a single transcript of approx. 1.9 kb and is expressed in all tissues examined at approximately equal levels. This is similar to the expression of  $\gamma 1$  in rat, which we and others have reported previously [15,16]. When the blot was probed with  $\gamma 2$  cDNA two

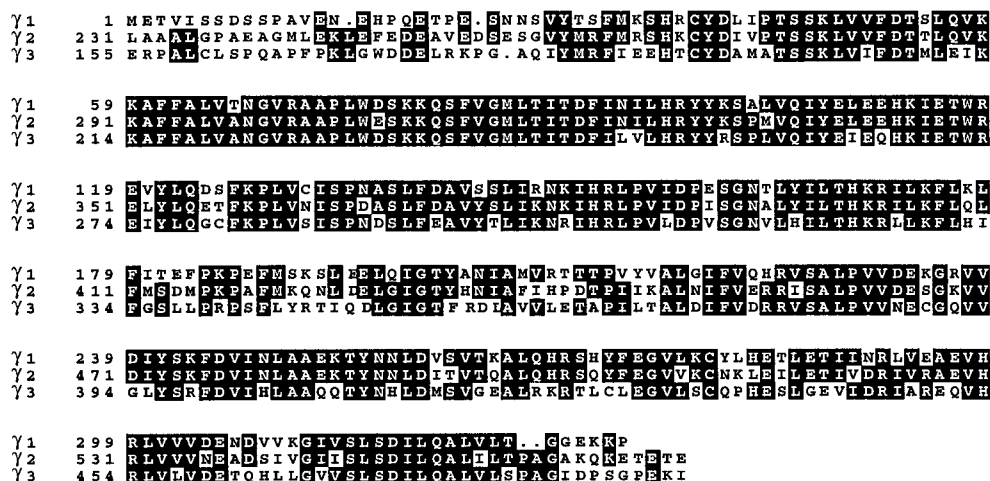
transcripts of approx. 3.8 kb and 2.4 kb were detected. In some tissues, such as brain and kidney, both transcripts were present. In heart, lung, placenta and skeletal muscle only the 2.4 kb transcript was detected, whereas in liver only the 3.8 kb message was seen. Using a  $\gamma 3$ -specific probe, a strong signal corresponding to a transcript of approx. 2.4 kb was detected in skeletal muscle with faint signals in heart and pancreas. A transcript of approx. 4.4 kb was also detected with the  $\gamma 3$  probe in all tissues examined, with highest levels again being found in skeletal muscle.

#### Interaction of the $\gamma$ isoforms with the $\alpha$ and $\beta$ subunits

We used the two-hybrid system in order to investigate the interaction of the various  $\gamma$  isoforms with the  $\alpha$  and  $\beta$  subunits. The results are shown in Table 1. The  $\gamma 1$  and  $\gamma 3$  isoforms interacted strongly with both the  $\beta 1$  and  $\beta 2$  isoforms, whereas the interaction of the  $\gamma 2$  isoform with the  $\beta$  isoforms was noticeably weaker. Unlike the  $\gamma 2$  and  $\gamma 3$  isoforms, the  $\gamma 1$  isoform produced a faint blue colouration when transformed with either  $\alpha 1$  or  $\alpha 2$ . No interaction could be detected between the  $\gamma$  isoforms themselves (results not shown).

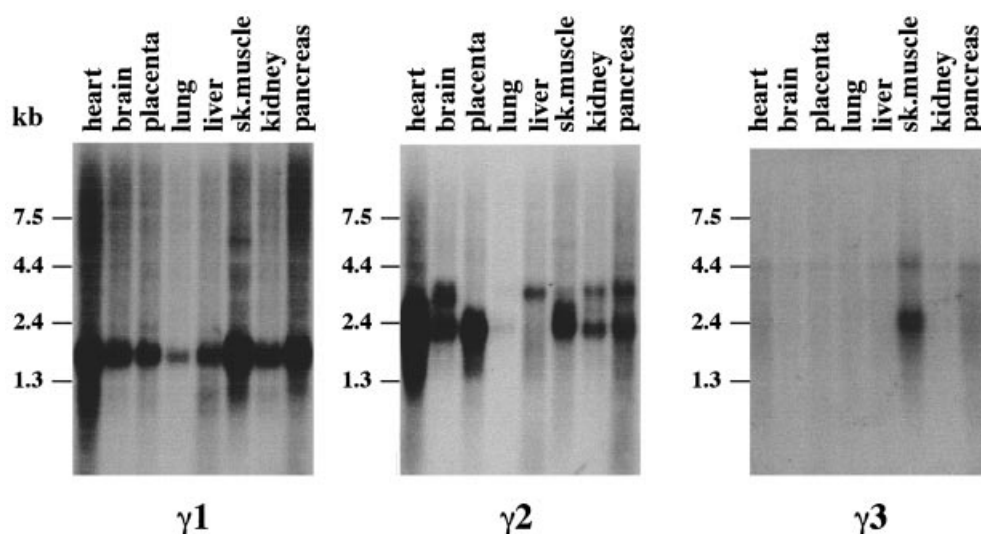
#### Tissue distribution of $\alpha$ and $\gamma$ isoforms assessed by immunoprecipitation

To allow biochemical studies of AMPK complexes containing different  $\gamma$  isoforms, we generated anti-peptide antibodies using sequences specific for each isoform (see the Experimental section). We assessed their effectiveness for Western blotting (results not shown) using a resuspended polyethylene glycol precipitate prepared from a rat liver extract. The anti- $\gamma 1$  antibody specifically recognized a polypeptide of the expected molecular mass (36 kDa). Unfortunately (for reasons that remain unclear) the antibodies against  $\gamma 2$  and  $\gamma 3$  recognized a number of polypeptides, none of which corresponded to the expected molecular masses of  $\gamma 2$  or  $\gamma 3$  (results not shown). However, all three antibodies were effective in immunoprecipitation studies. To



**Figure 4** Comparison of the amino acid sequences of human AMPK $\gamma$  isoforms

The deduced amino acid sequences of  $\gamma 2$  (residues 231–569) and  $\gamma 3$  (residues 155–492) were aligned with the entire coding sequence of  $\gamma 1$  using the GAP program (University of Wisconsin package), with a gap weight of 3.0 and a length weight of 0.1. Dots indicate gaps introduced to maximize the alignment. Identities between two or all three sequences are boxed. The N-terminal 230 amino acids of  $\gamma 2$  and 154 amino acids of  $\gamma 3$  show no significant identity with one another and are not included.



**Figure 5** Northern-blot analysis of  $\gamma$ -subunit isoforms

A human multiple-tissue Northern blot (ClonTech) was hybridized with a cDNA probe encoding either  $\gamma 1$ ,  $\gamma 2$  or  $\gamma 3$ . In each case the blot was washed with  $0.2 \times \text{SSC}/0.5\% \text{SDS}$  at  $60^\circ \text{C}$  and exposed at  $-70^\circ \text{C}$ . Migration of RNA standards is indicated.

**Table 1** Interactions between the  $\gamma$  isoforms and the  $\alpha$  and  $\beta$  subunits

Yeast (strain SFY526) was co-transformed with vectors expressing fusions of the various AMPK subunit isoforms with either the GAL4 DNA-binding domain ( $G_{BD}$ ) or the GAL4 activation domain ( $G_{AD}$ ). Several colonies from each transformation were grown and transferred to nitrocellulose in order to test for interactions by  $\beta$ -galactosidase filter assay. Interactions were classified as strong ( $++$ ), moderate ( $+$ ), weak ( $\pm$ ), or undetectable ( $-$ ) based on the intensity of the blue colouration that developed. The time taken for the colouration to appear (in min) is shown in parentheses.

DNA-binding hybrid	Activation-hybrid	Interaction (min before blue colouration)
$G_{BD}\text{-}\gamma 1$	$G_{AD}\text{-}\alpha 1$	$\pm$ ( $> 90$ )
$G_{BD}\text{-}\gamma 1$	$G_{AD}\text{-}\alpha 2$	$\pm$ ( $> 90$ )
$G_{BD}\text{-}\gamma 1$	$G_{AD}\text{-}\beta 1$	$++$ (30)
$G_{BD}\text{-}\gamma 1$	$G_{AD}\text{-}\beta 2$	$++$ (15)
$G_{BD}\text{-}\gamma 2$	$G_{AD}\text{-}\alpha 1$	$-$
$G_{BD}\text{-}\gamma 2$	$G_{AD}\text{-}\alpha 2$	$-$
$G_{BD}\text{-}\gamma 2$	$G_{AD}\text{-}\beta 1$	$\pm$ ( $> 90$ )
$G_{BD}\text{-}\gamma 2$	$G_{AD}\text{-}\beta 2$	$+$ (90)
$G_{BD}\text{-}\gamma 3$	$G_{AD}\text{-}\alpha 1$	$-$
$G_{BD}\text{-}\gamma 3$	$G_{AD}\text{-}\alpha 2$	$-$
$G_{BD}\text{-}\gamma 3$	$G_{AD}\text{-}\beta 1$	$++$ (45)
$G_{BD}\text{-}\gamma 3$	$G_{AD}\text{-}\beta 2$	$++$ (15)

confirm their specificity, we carried out successive immunoprecipitations of the same rat liver extract with the three antibodies in turn, and measured the amount of activity depleted from the supernatant after each precipitation step. Irrespective of the order in which the antibodies were added (all six combinations were tested), the anti- $\gamma 1$  antibody precipitated 85–90%, the anti- $\gamma 2$  antibody precipitated 8–12%, and the anti- $\gamma 3$  antibody precipitated 2–3% of the starting activity. Between them, the three antibodies depleted 99–100% of the starting activity (Table 2).

We then assessed the tissue distribution of each  $\gamma$ -subunit isoform by measuring what proportion of total AMPK activity

**Table 2** Successive immunoprecipitation of  $\gamma$  isoforms from a rat liver extract (resuspended polyethylene glycol precipitate)

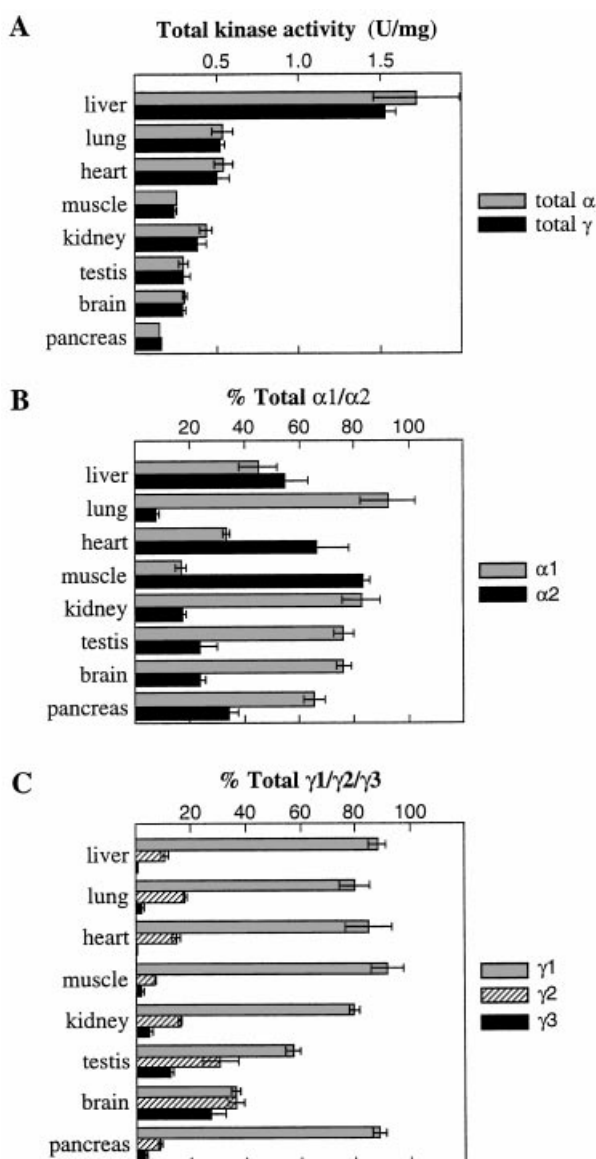
The same proportion of activity was recovered in the pellet using each anti- $\gamma$ -subunit antibody, irrespective of the order in which successive immunoprecipitations were performed. The results are expressed as % of the initial starting activity recovered in each immunoprecipitate and (in the right-hand column) the total recovered in all three immunoprecipitates.

Order of immunoprecipitation	$\gamma 1$	$\gamma 2$	$\gamma 3$	Total
$\gamma 1, \gamma 2, \gamma 3$	90	7.7	2.0	99.7
$\gamma 2, \gamma 1, \gamma 3$	85	12	1.9	98.9
$\gamma 3, \gamma 2, \gamma 1$	89	7.9	2.9	99.8
$\gamma 1, \gamma 3, \gamma 2$	90	8.6	1.7	100.3
$\gamma 2, \gamma 3, \gamma 1$	87	9.8	1.7	98.5
$\gamma 3, \gamma 1, \gamma 2$	88	9.8	1.7	99.5
Mean $\pm$ S.D.	$88 \pm 2$	$9.3 \pm 1.6$	$2.0 \pm 0.5$	$99.4 \pm 0.7$

was immunoprecipitated with each antibody. We also compared the distribution of the  $\alpha 1$  and  $\alpha 2$  isoforms in the same tissue extracts by immunoprecipitation, using the  $\alpha 1$ - and  $\alpha 2$ -specific antibodies raised previously [29].

In all tissues examined, immunoprecipitation with a combination of anti- $\alpha 1$  and anti- $\alpha 2$  antibodies depleted essentially 100% of the AMPK activity (proportion depleted: liver, 98%; lung, 98%; heart, 98%; muscle, 100%; kidney, 97%; testis, 99%; brain, 100%; pancreas, 98%). This indicates that, if additional isoforms of the  $\alpha$  subunit exist, they do not account for a significant fraction of the AMPK activity in these tissues.

Figure 6(A) shows that the total activity recovered in immunoprecipitates using anti- $\gamma 1$ , - $\gamma 2$  and - $\gamma 3$  was also the same as the total activity recovered with anti- $\alpha 1$  and - $\alpha 2$ . There are, therefore, no additional  $\gamma$ -subunit isoforms that contribute significantly to total AMPK activity in these tissue extracts.



**Figure 6** Tissue distribution of AMPK activity assessed using immunoprecipitation

(A) Total AMPK activity immunoprecipitated (units per mg of extract protein) using anti- $\alpha 1$ - $\alpha 2$  antibodies, and anti- $\gamma 1$ - $\gamma 2$ - $\gamma 3$  antibodies, from the eight tissues tested. (B) Proportion of the total AMPK activity immunoprecipitated using anti- $\alpha$  antibodies that was precipitated by anti- $\alpha 1$  or anti- $\alpha 2$  antibodies alone. (C) Proportion of the total AMPK activity immunoprecipitated using anti- $\gamma$  antibodies that was precipitated by anti- $\gamma 1$ , anti- $\gamma 2$  or anti- $\gamma 3$  antibodies alone. Results are expressed as the means  $\pm$  S.E.M. of three separate determinations.

Figure 6(B) shows the distribution of  $\alpha$ -subunit isoforms assessed by immunoprecipitation. As reported previously [29],  $\alpha 1$  and  $\alpha 2$  complexes each accounted for about half of the total AMPK activity in rat liver extracts. We have now extended the analysis to a broader range of extracts. In lung, kidney, testis and brain,  $\alpha 1$  complexes account for the major part (60–90%) of total AMPK activity, with  $\alpha 2$  complexes accounting for the remaining 10–40%. In contrast,  $\alpha 2$  complexes account for the major part (70–80%) of total AMPK activity in extracts of cardiac and skeletal muscle, with  $\alpha 1$  complexes making up the remaining 20–30%. This is in broad agreement with qualitative

studies performed by Western blotting, where  $\alpha 2$  was found to be particularly abundant in cardiac and skeletal muscle [17].

Figure 6(C) shows the distribution of  $\gamma$ -subunit isoforms assessed by immunoprecipitation.  $\gamma 1$  complexes accounted for the largest part of AMPK activity in all tissue extracts examined, followed by  $\gamma 2$ , with  $\gamma 3$  accounting for least. In extracts of liver, lung, cardiac muscle, skeletal muscle, kidney and pancreas,  $\gamma 1$  complexes account for 80–90% of the total activity, with  $\gamma 2$  accounting for only 10–20%, and  $\gamma 3$  having only a very minor activity. The situation was different in testis and brain, where the three  $\gamma$ -subunit isoforms made more equal contributions to total AMPK activity, especially in the brain, where the proportions of  $\gamma 1$ ,  $\gamma 2$  and  $\gamma 3$  were 36%, 36% and 27% respectively.

#### $\gamma$ Isoforms do not associate with specific $\alpha$ subunits

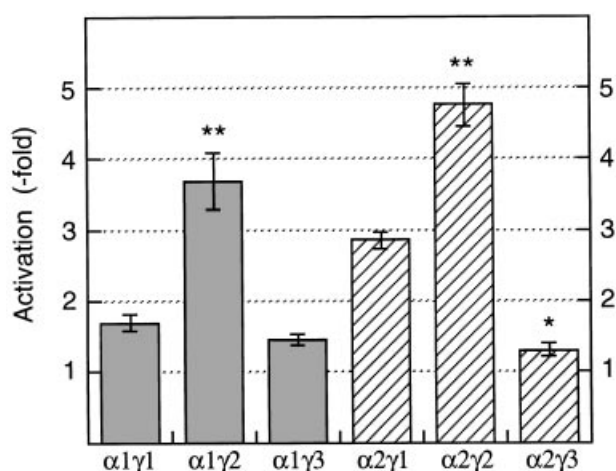
Previous work has suggested that  $\alpha 1$  or  $\alpha 2$  do not have a preference for  $\beta 1$  or  $\beta 2$  [18]. An important question was to determine whether  $\gamma 1$ ,  $\gamma 2$  or  $\gamma 3$  show any preference for association with either the  $\alpha 1$  or  $\alpha 2$  isoform of the catalytic subunit *in vivo*. To address this, we prepared partially purified rat liver extracts containing specifically either  $\alpha 1$  or  $\alpha 2$  complexes, by first depleting the other isoform by immunoprecipitation. Each extract was then split into three, immunoprecipitated with  $\gamma 1$ ,  $\gamma 2$  or  $\gamma 3$  antibody, and the kinase activity in the pellets determined. Irrespective of whether the  $\alpha 1$  or  $\alpha 2$  fractions were analysed, the  $\gamma 1$ ,  $\gamma 2$  and  $\gamma 3$  isoforms accounted for the same proportion of the total activity. The  $\gamma 1$  subunit accounted for  $82 \pm 6\%$  ( $\alpha 1$ ) or  $81 \pm 5\%$  ( $\alpha 2$ ), the  $\gamma 2$  subunit for  $16 \pm 3\%$  ( $\alpha 1$ ) or  $15 \pm 4\%$  ( $\alpha 2$ ), and the  $\gamma 3$  subunit for  $2 \pm 3\%$  ( $\alpha 1$ ) or  $2 \pm 1\%$  ( $\alpha 2$ ) of the activity recovered (mean  $\pm$  S.E.M. for 3 experiments). This suggests that all six combinations of  $\alpha$  and  $\gamma$  subunits ( $\alpha 1\gamma 1$ ,  $\alpha 1\gamma 2$ ,  $\alpha 1\gamma 3$ ,  $\alpha 2\gamma 1$ ,  $\alpha 2\gamma 2$ ,  $\alpha 2\gamma 3$ ) can form equally readily, without any selective association of distinct  $\alpha$  and  $\gamma$  isoforms.

#### Different $\alpha\gamma$ complexes have different degrees of dependence on AMP

We next addressed whether complexes containing different combinations of  $\alpha$  and  $\gamma$  subunits might differ in their response to AMP. For this study we used rat brain extracts, in which there were almost equal proportions of the  $\gamma 1$ ,  $\gamma 2$  and  $\gamma 3$  isoforms. We prepared extracts containing only  $\alpha 1$  or  $\alpha 2$  complexes (by immunoprecipitation of the other isoform, and then made immunoprecipitates using anti- $\gamma 1$ , anti- $\gamma 2$ , or anti- $\gamma 3$  antibodies as above. Each immunoprecipitate was assayed with or without AMP. The results showed that the AMP dependence of the complex depended on the nature of the  $\gamma$  subunit as well as the  $\alpha$  subunit (Figure 7). Consistent with previous results, where the distinct  $\gamma$  isoforms had not been separated [20],  $\alpha 2$  complexes were more AMP-dependent than the equivalent  $\alpha 1$  complexes (with the exception of complexes containing  $\gamma 3$ ). Figure 7 reveals that  $\gamma 2$  complexes are more AMP-dependent than  $\gamma 1$  complexes. This was particularly striking for the  $\alpha 1$  complexes, where  $\alpha 1\gamma 2$  complexes were stimulated 3.7-fold against only 1.7-fold for  $\alpha 1\gamma 1$  complexes. The  $\gamma 3$  complexes give a rather low AMP-dependence in every case ( $\alpha 1\gamma 3$ , 1.5-fold;  $\alpha 2\gamma 3$ , 1.3-fold).

Very similar results were obtained in experiments where the complexes were immunoprecipitated from extracts of rat liver, although we could be less confident about the results obtained for the  $\alpha 1\gamma 3$  and  $\alpha 2\gamma 3$  complexes due to the low abundance of the  $\gamma 3$  isoform in rat liver.

We also investigated the possibility that there were differences in AMP sensitivity by immunoprecipitating the  $\gamma 1$  and  $\gamma 2$



**Figure 7** AMP-dependence of different AMPK complexes

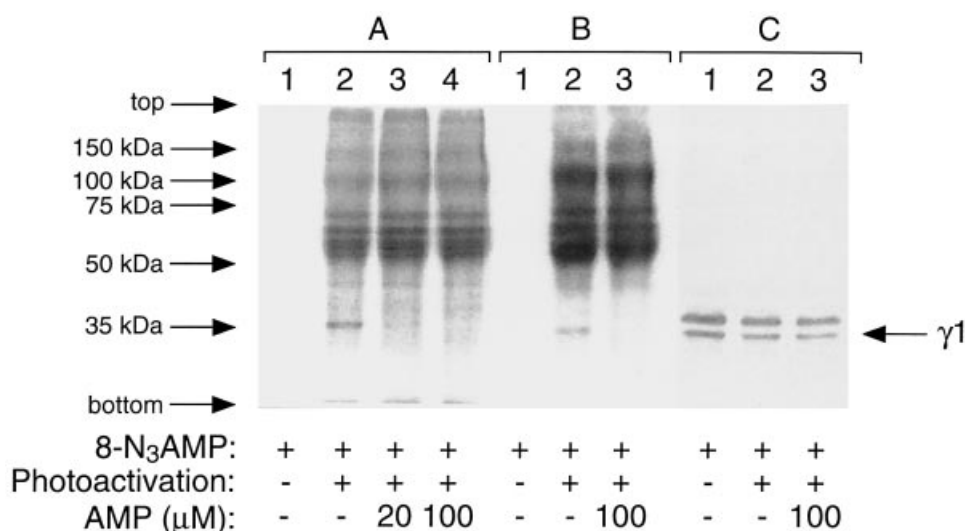
An extract from a single rat brain containing specifically  $\alpha 1$ - or  $\alpha 2$  complexes (prepared by immunodepletion of the other isoform) was immunoprecipitated with anti- $\gamma 1$ ,  $\gamma 2$  or  $\gamma 3$  antibodies. After extensive washing, AMPK activity in each pellet was measured in the presence or absence of 200  $\mu\text{M}$  AMP. The results (means  $\pm$  S.E.M. of three separate determinations) are expressed as the ratio of the activities in the presence or absence of AMP. Asterisks indicate cases where the AMP-dependence was significantly different from that of the  $\gamma 1$  complex with the same  $\alpha$ -subunit isoform (\* $P < 0.01$ ; \*\* $P < 0.001$ ). Statistical significances were tested by one-way analysis of variance using Bonferroni's multiple comparison test.

complexes from crude rat liver extracts (without prior separation of  $\alpha 1$  and  $\alpha 2$ ), and assaying at different concentrations of AMP. This revealed that  $\gamma 1$  and  $\gamma 2$  complexes had indistinguishable  $\text{EC}_{50}$  values for AMP of approx. 5  $\mu\text{M}$  (results not shown).

### Evidence that the AMP-binding site involves the $\gamma$ subunit

The findings in the previous section showed that the identity of the  $\gamma$  subunit, as well as the  $\alpha$  subunit, affected the AMP-dependence of the AMPK complex, suggesting that the allosteric AMP-binding site might involve the  $\gamma$  subunit, as well as the  $\alpha$  subunit. In order to address this further, we utilized the photoaffinity analogue 8-azido-AMP. In this compound the reactive group is on the adenine moiety rather than in the position equivalent to the  $\gamma$  phosphate of ATP, as in *p*-fluorosulphonyl-benzoyl-adenosine (FSBA) which was used previously to infer the involvement of the  $\alpha$  subunit in the binding of AMP [31]. In the absence of photoactivation by UV light, 8-azido-AMP acted as a partial agonist. Addition of 8-azido-AMP to the assay caused a small stimulation of AMPK activity, with the maximal effect being only approx. 25% of that given by AMP itself. The  $\text{EC}_{50}$  for this activating effect was  $10 \pm 3 \mu\text{M}$ . The presence of 100  $\mu\text{M}$  8-azido-AMP also antagonized activation by AMP, with the  $\text{EC}_{50}$  for the effect of AMP increasing from 5  $\mu\text{M}$  to 170  $\mu\text{M}$ . These results indicate that 8-azido-AMP binds at the allosteric site, but only partially mimics the ability of AMP to produce the active conformation.

We then synthesized 8-azido- $^{32}\text{P}$ AMP, and studied its covalent binding to the subunits of AMPK after photoactivation. We utilized a preparation of rat liver AMPK purified as far as the gel filtration step [26], in which the  $\beta 1$  and  $\gamma 1$  subunits can be clearly resolved by SDS/PAGE, but in which there are contaminants in the 50–100 kDa range which obscure the  $\alpha$  subunits (and  $\gamma 2$  and  $\gamma 3$ ). 8-Azido- $^{32}\text{P}$ AMP was allowed to bind to the preparation and the mixture was then flashed with UV light to cause photoactivation. The results (Figure 8) showed that the reaction was rather non-specific, with many polypeptides becoming  $^{32}\text{P}$ -labelled. However, if AMP was included in the initial incubation with 8-azido- $^{32}\text{P}$ AMP, the labelling of a prominent band at 36 kDa was almost abolished at 20  $\mu\text{M}$ , and



**Figure 8** Photoaffinity labelling of partially purified AMPK using 8-azido- $^{32}\text{P}$ AMP

AMPK was purified as far as the gel-filtration step. On a Coomassie-stained SDS gel the  $\beta 1$  and  $\gamma 1$  subunits were well resolved but contaminants in the 50–100 kDa range obscured the  $\alpha$  subunit (results not shown). The preparation was incubated with 8-azido- $^{32}\text{P}$ AMP in the presence or absence of AMP (20  $\mu\text{M}$  and 100  $\mu\text{M}$ ), flashed with UV light, and analysed by SDS/PAGE. (A) Dried gel subject to autoradiography; (B) a similar experiment except proteins were transferred to nitrocellulose and the filter subjected to autoradiography; (C) the same experiment as (B), but the blot was probed with an anti- $\gamma 1$  antibody. The anti- $\gamma 1$  antibody used (not the same as that used in other experiments) was raised against a glutathione S-transferase fusion protein containing the entire  $\gamma 1$  sequence [20]. As well as  $\gamma 1$ , this antibody detects an unidentified polypeptide migrating on SDS/PAGE with an apparent molecular mass of approx. 40 kDa.



completely abolished at 100  $\mu$ M AMP (Figure 8). The labelling of the other polypeptides in the preparation was completely unaffected by the presence of 100  $\mu$ M AMP. Western blotting using an anti- $\gamma$ 1 antibody confirmed that the 36 kDa polypeptide, whose labelling was protected by AMP, was the  $\gamma$ 1 subunit (Figure 8).

## DISCUSSION

We report here the identification of two novel isoforms of the AMPK  $\gamma$  subunit.  $\gamma$ 2 cDNA was identified by interrogation of the EST database using the amino acid sequence of human  $\gamma$ 1. In a previous study, Stapleton et al. reported a partial amino acid sequence for  $\gamma$ 2 (corresponding to residues 318–569 of full length  $\gamma$ 2), also identified by database searching [30]. As part of a study aimed at identifying proteins which interact with the  $\beta$  subunit of AMPK we identified a third isoform,  $\gamma$ 3, from a human skeletal muscle two-hybrid library. The polypeptides encoded by  $\gamma$ 2 and  $\gamma$ 3 are predicted to be much larger than  $\gamma$ 1, and translation of the isoforms *in vitro* supports this view (Figure 3). Sequence alignment of the isoforms reveals that this difference in size is due to the fact that both  $\gamma$ 2 and  $\gamma$ 3 have a long N-terminal extension that is not present in  $\gamma$ 1. The function of the N-terminal domains of  $\gamma$ 2 and  $\gamma$ 3 is not known at present, although it is interesting to note that  $\gamma$ 2 contains a putative bipartite nuclear localization signal [32] between residues 9–24. Previously, it was shown that the  $\gamma$ 1 subunit interacts strongly with the  $\beta$ 1 subunit, but only very weakly with the  $\alpha$ 2 subunit [15]. We did not find any significant differences in the interactions of the  $\gamma$ -subunit isoforms with the  $\alpha$  and  $\beta$  subunits, as assessed by the two-hybrid system (Table 1). It seems unlikely, therefore, that the  $\gamma$  isoforms cause any selective association between the subunits within the complex (discussed further below).

$\gamma$ 2 mRNA, like  $\gamma$ 1 mRNA [15,16], shows a broad tissue distribution, whereas significant expression of  $\gamma$ 3 mRNA was found only in skeletal muscle from the tissues we examined. We have shown previously that  $\alpha$ 2 mRNA [19] and  $\beta$ 2 mRNA [18] are highly expressed in skeletal muscle, whereas the  $\alpha$ 1 and  $\beta$ 1 isoforms are more uniformly expressed in rat tissues [15,17,24]. We were unable to investigate the tissue distribution of the  $\gamma$  isoform proteins directly, since the anti- $\gamma$ 2 and - $\gamma$ 3 antibodies we raised did not recognize polypeptides of the expected molecular mass on Western blots. The antibodies did immunoprecipitate AMPK activity from tissue extracts, however, and we were able to use this property to obtain an indirect measurement of the expression of the  $\gamma$  isoforms. The results in Figure 6(A), which are estimates of AMPK activities in various rat tissue extracts obtained by measuring the activities in anti- $\alpha$  subunit or anti- $\gamma$  subunit immunoprecipitations, are in broad agreement with those obtained previously using peptide kinase assays in crude extracts [27]. The highest activity (approx. 1.5 units/mg) was found in liver, with the activity in most other extracts being lower (0.2–0.5 units/mg). A caveat with these studies is that we cannot be sure whether the degree of activation of the kinase is the same in all extracts. In rat liver, the kinase becomes activated by *post mortem* increases in the AMP:ATP ratio, presumed to be caused by hypoxia [33]. This may not occur to the same extent in other tissues, particularly in muscle where phosphocreatine would buffer the AMP:ATP ratio.

In all of the tissue extracts examined for Figure 6, a combination of anti- $\alpha$ 1 and anti- $\alpha$ 2 antibodies immunoprecipitated all of the AMPK activity, with none remaining in the supernatant. Figure 6(A) shows that the total activity immunoprecipitated by anti- $\gamma$ 1, - $\gamma$ 2 and - $\gamma$ 3 antibodies was essentially identical to that brought down by the anti- $\alpha$  antibodies. This shows that if

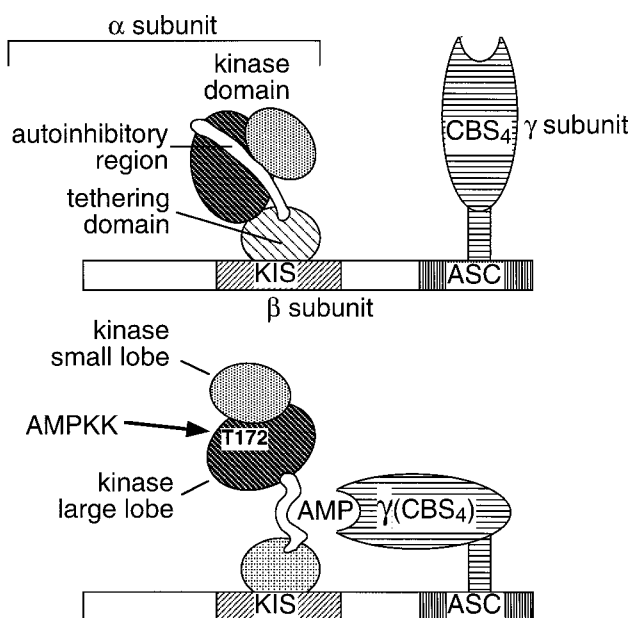
additional  $\gamma$ -subunit isoforms exist, they do not contribute a significant amount of AMPK activity in the tissue extracts examined.

Figure 6(B) shows that, in terms of the proportion of total AMPK activity measured,  $\alpha$ 2 is the predominant isoform in cardiac and skeletal muscle,  $\alpha$ 1 is predominant in lung, kidney, testis, brain and pancreas while (as reported previously [29]) the  $\alpha$ 1- and  $\alpha$ 2-isoforms make approximately equal contributions in rat liver. This is in broad agreement with the qualitative results of Western blotting [17], but contradicts previous claims that  $\alpha$ 2 complexes contributed only a small proportion of AMPK activity in rat liver extracts [17]. Figure 6(C) shows that the distribution of  $\gamma$ -subunit isoforms appears to be more uniform. In most tissues examined,  $\gamma$ 1 was the predominant isoform, with  $\gamma$ 2 being less abundant, and  $\gamma$ 3 being only a minor isoform. Exceptions to this were testis and brain, particularly the latter, where the contribution of  $\gamma$ 1,  $\gamma$ 2 and  $\gamma$ 3 was approximately equal. The brain is of course very heterogeneous, and it will be interesting to examine whether any specific cell types in the brain express  $\gamma$ 2 or  $\gamma$ 3 as the predominant isoform.

The tissue distribution of the  $\gamma$ 1 and  $\gamma$ 2 isoforms, as determined by immunoprecipitation, are consistent with the widespread tissue distribution of mRNA coding for these polypeptides (Figure 5). However, this was not the case for the  $\gamma$ 3 isoform. Northern-blot analysis of the  $\gamma$ 3 subunit showed very high-level expression in skeletal muscle relative to the levels detected in the other tissues examined. In contrast, the results of the immunoprecipitation experiments indicated that AMPK activity associated with this isoform was highest in brain and testis respectively, with only very low activities being detected in the other tissues, including skeletal muscle. A difficulty is that we were unable to examine directly the level of protein expression for the  $\gamma$  isoforms. It is possible, therefore, that although the activity associated with the  $\gamma$ 3 subunit is very low in some tissue extracts, the actual protein expression may be higher. There could be a number of reasons for this: (i)  $\gamma$ 3-containing complexes might be present in an inactive form; (ii)  $\gamma$ 3 might be expressed in the absence of the other subunits; (iii)  $\gamma$ 3-containing complexes might be present in the particulate fraction, rather than the soluble fraction (15000 *g* supernatant) that was used as the source for immunoprecipitation.

Our results indicate that there is no selective association between  $\alpha$ 1 and  $\alpha$ 2 and the  $\gamma$ 1,  $\gamma$ 2 or  $\gamma$ 3 subunits. Previous work had suggested that there are no selective associations between the  $\alpha$ 1 and  $\alpha$ 2 subunits and the  $\beta$ 1 and  $\beta$ 2 subunits either [18]. It remains unclear at present why in some cells there appear to be two pools of AMPK complexes containing the  $\alpha$ 2 subunit, i.e. a cytoplasmic pool and a nuclear pool [20]. Although our data do not support the idea that there are additional  $\alpha$ - and  $\gamma$ -subunit isoforms, we cannot yet rule out the possible existence of additional  $\beta$ -subunit isoforms. The  $\beta$  subunits are perhaps the best candidates to have a targeting role, since they appear to be the scaffold on which the  $\alpha$  and  $\gamma$  subunits assemble [2,15,34]. There is also some genetic evidence that the three distinct  $\beta$  subunits in yeast have targeting roles [2].

We have shown previously that  $\alpha$ 2 complexes purified from rat liver by immunoprecipitation were stimulated to a much larger extent by AMP (approx. 5-fold) than were  $\alpha$ 1 complexes (approx. 2-fold), although their sensitivity to different concentrations of AMP was similar [20]. However, it is now clear that the  $\alpha$ 1 and  $\alpha$ 2 complexes used in these studies would have been heterogeneous mixtures containing different  $\gamma$ -subunit isoforms. The results in Figure 7 now show that the identity of the  $\gamma$  isoform present in the complex also has a marked influence on the AMP-dependence. Complexes containing  $\gamma$ 2 gave a higher AMP-



**Figure 9** Model for the regulation of the AMPK complex

In the absence of AMP, the complex exists predominantly in the inactive conformation shown in the top panel, where the  $\alpha$ - and  $\gamma$ -subunits do not interact (except indirectly via the  $\beta$  subunit). In this state, phosphorylation of Thr<sup>172</sup> and access to exogenous substrates is blocked by interactions between the catalytic cleft of the kinase domain and the autoinhibitory region on the  $\alpha$  subunit. In the active conformation (lower panel), interaction between the catalytic cleft and the autoinhibitory region is prevented by interaction of the latter with one or more of the CBS domains of the  $\gamma$  subunit. The interaction between the autoinhibitory region and the  $\gamma$  subunit is stabilized by binding of AMP, which involves residues on both subunits.

dependence than those containing  $\gamma 1$ , a difference that was most marked for the less AMP-dependent  $\alpha 1$  complexes. We could not detect any difference in sensitivity (the  $EC_{50}$ ) for AMP. Complexes containing  $\gamma 3$  gave a rather low AMP-dependence with either  $\alpha$  subunit.

The results in Figure 8 also suggest that the  $\gamma$  subunits may be directly involved in the AMP-binding site. Previous studies on the location of the AMP-binding site utilized the reactive ATP analogue, FSBA, which binds at both the allosteric (AMP/ATP-binding) and the catalytic (ATP-binding) sites [31]. Experiments using [<sup>14</sup>C]FSBA showed that it reacted exclusively with the  $\alpha$  subunit [31], which suggested that the allosteric site was located on the  $\alpha$  subunit, but did not rule out the possibility that the  $\beta$  and/or  $\gamma$  subunits were also involved. We investigated the use of a photoaffinity analogue of AMP, 8-azido-AMP, where the reactive group is at the opposite end of the nucleotide to that on FSBA. In the absence of photoactivation, 8-azido-AMP acted as a partial agonist, indicating that the analogue does indeed bind at the allosteric site for AMP. When we used 8-azido-[<sup>32</sup>P]AMP and flashed with UV light, the reagent appeared to act rather non-specifically, and several polypeptides in the preparation became labelled. However, the labelling of the  $\gamma 1$  subunit was protected specifically and completely by concentrations of AMP that would saturate the allosteric site on AMPK. Taken together with previous studies showing labelling of the  $\alpha$  subunit by [<sup>14</sup>C]FSBA, this provides strong evidence that the AMP-binding site lies between the  $\alpha$  and  $\gamma$  subunits.

Figure 9 shows a model for the regulation of the AMPK complex which takes into account our results, the two-hybrid analysis of the yeast SNF1 complex by Jiang and Carlson [35],

and studies with truncated forms of the  $\alpha 1$  subunit by Crute et al. [23]. In the inactive state (Figure 9, top panel), the  $\beta$  subunit is the scaffold via which the complex assembles, with the  $\alpha$  subunit binding to its KIS domain and the  $\gamma$  subunit binding to its ASC domain. Under these conditions the  $\alpha$  and  $\gamma$  subunits do not interact directly. Although originally based on the two-hybrid analysis with yeast SNF1 [35], this part of the model is consistent with the findings that  $\beta 1$  and  $\beta 2$  are most highly conserved with the yeast  $\beta$  subunits within the KIS and ASC domains [18]. It is also consistent with previous findings that the mammalian  $\alpha$  and  $\gamma$  subunits do not interact by two-hybrid analysis in yeast, or when co-expressed (without  $\beta$ ) in reticulocyte lysates [15]. In the yeast system one would expect AMP to be low since the yeast were maintained in high glucose, whereas in the reticulocyte lysate the AMP would be low because of the inclusion of an ATP-regenerating system. Under these conditions the model proposes that  $\alpha$  and  $\gamma$  do not interact directly, but only indirectly via the  $\beta$  subunit.

The model also proposes that in the absence of AMP the kinase domain is inactive because of association with an autoinhibitory domain which blocks the active site cleft between the small and large lobes of the kinase domain, and prevents access of AMPKK to the activating Thr<sup>172</sup> site. The evidence for this comes from two-hybrid analysis in yeast under conditions where the kinase is inactive (high glucose), when a region from residues 392 to 518 in the regulatory domain of Snf1p was shown to interact with the kinase domain [35]. It is also consistent with recent studies of expression of truncated forms of  $\alpha 1$  [23], which suggested that a region from 313–392 on  $\alpha 1$  contains an autoinhibitory segment. This region is equivalent to residues 368–494 of Snf1p.

Based on the results in this paper, the model also proposes that AMP binds between the  $\alpha$  and  $\gamma$  subunits, stabilizing their association and thus preventing the interaction of the autoinhibitory domain with the active site. This in turn exposes Thr<sup>172</sup> for phosphorylation by AMPKK, leading to the activation of the kinase. The exact location of the AMP-binding site within the AMPK  $\alpha$  and  $\gamma$  subunits remains unknown, but Jiang and Carlson [35] have shown that Snf1p and Snf4p (equivalent to  $\alpha$  and  $\gamma$ , respectively) interact under derepressing (SNF1-activating) conditions via the region from residues 392–495 on Snf1p. This overlaps with the region (392–518) containing the putative autoinhibitory domain on Snf1p, and it therefore seems likely that the interaction of the  $\alpha$  subunit with the  $\gamma$  subunit, and the interaction of the kinase domain with the autoinhibitory domain, would be mutually exclusive.

In our model, the binding of AMP stabilizes the interaction between  $\alpha$  and  $\gamma$  by cross-linking the two subunits. The AMP-dependence of specific complexes (e.g.  $\alpha 1$ - $\gamma 1$ , 2-fold versus  $\alpha 2$ - $\gamma 2$ , 5-fold) would depend on the relative stabilities of the  $\alpha \leftrightarrow \gamma$  subunit interaction and the kinase  $\leftrightarrow$  autoinhibitory domain interaction, and on the degree of stabilization of the former by AMP.

Although the AMPK  $\gamma$  subunit was originally reported to be unrelated to other proteins in the database (with the exception of yeast Snf4p), Bateman [36] recently reported that it comprises four repeats of a newly defined protein module called a cystathionine  $\beta$ -synthase (CBS) domain. The functions of CBS domains are not known, although they appear to be regulatory rather than catalytic in function [36]. The enzyme CBS is involved in the pathway of synthesis of cysteine from homocysteine, and has a single CBS domain. Mutations in the gene encoding this enzyme give rise to homocystinuria, and at least one human mutation (D444N) occurs in the CBS domain. This mutation does not affect the basal catalytic activity, but instead abolishes

the activation by *S*-adenosylmethionine [37]. This raises the intriguing possibility that one function of CBS domains might be to bind adenosine derivatives, such as *S*-adenosylmethionine in cystathionine  $\beta$ -synthase and AMP in AMPK.

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