The α1 and α2 isoforms of the AMP-activated protein kinase have similar activities in rat liver but exhibit differences in substrate specificity in vitro

Angela Woods[a], Ian Salt[b], James Scott[a], D. Grahame Hardie[b], David Carling[a,*]

[a]*MRC Molecular Medicine, Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road, London W12 0NN, UK*
[b]*Department of Biochemistry, The University, Dundee, DD1 4HN, UK*

Received 7 October 1996

Abstract The AMP-activated protein kinase (AMPK) is a heterotrimeric complex composed of a catalytic subunit (α) and two regulatory subunits (β and γ). Two isoforms of the catalytic subunit (α1 and α2) have been identified. We show here that the α1- and α2-containing complexes contribute approximately equally to total AMPK activity in rat liver. Furthermore, expression of α1 or α2 with β and γ in mammalian cells demonstrates that both complexes have equal specific activity measured with the SAMS peptide. Using variant peptides, however, we show that α1 and α2 exhibit slightly different substrate preferences, which suggest that the two isoforms could play different physiological roles within the cell.

Key words: AMP-activated protein kinase; Subunit isoform; Specificity determinant; Consensus sequence

1. Introduction

The AMP-activated protein kinase (AMPK) plays a role in the cellular response to environmental stresses which deplete ATP [1,2]. AMPK, a heterotrimeric enzyme composed of a catalytic subunit (α) and two regulatory subunits (β and γ) [3,4], is activated by AMP via a number of independent mechanisms [5,6]. The α subunit is phosphorylated by an upstream protein kinase (AMPKK) resulting in greater than 50-fold activation of the AMPK complex [7]. Proteins related to all three subunits have been identified in the yeast SNF1 kinase complex [3,4,8,9] which is involved in derepression of glucose-repressed genes [10,11]. Plant cDNAs encoding proteins related to the catalytic subunit of AMPK and Snf1p have also been isolated [8,12]. It appears therefore that the AMPK-related protein kinase family is widely distributed and that they have been highly conserved throughout evolution.

A second form of AMPKα, encoded by a separate gene, has been identified recently [13]. It was reported that this isoform, which the authors termed α1, accounted for greater than 90% of AMPK activity present in rat liver measured using the SAMS peptide, whereas the α2 isoform, although present, accounted for less than 10% of the activity.

We have re-examined the activities of the α1- and α2-containing complexes in rat liver and find that each isoform contributes approximately equally to AMPK activity. Furthermore, expression of α1- or α2-containing complexes in a heterologous system resulted in similar levels of AMPK activity. Using a panel of synthetic peptides [14], we show quantitative differences in rates of phosphorylation by α1 and α2. These results imply that both isoforms are equally important in the AMPK response in rat liver, but that their specificity for downstream targets within the cell could differ.

*Corresponding author. Fax: (44) (181) 383 2028.
E-mail: dcarling@rpms.ac.uk

Abbreviations: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; SAMS, synthetic peptide substrate HMRSAMSGLHLVKRR; SDS-PAGE, SDS-polyacrylamide gel electrophoresis

2. Materials and methods

AMPK was purified from rat liver as previously described [15]. AMPKα antibodies were raised in sheep against isoform-specific peptides derived from the deduced amino acid sequences of α1 (TSPPDSFLDDHHLTR) or α2 (MDDSAMHIPPGLKPH). Peptides were synthesised with a cysteine residue at the N-terminus, coupled to keyhole limpet haemocyanin and used for immunisation. Antibodies were affinity purified from serum using the appropriate peptide conjugated to thiol-Sepharose [16]. Production of β and γ antibodies have been described elsewhere [4]. Protein G-Sepharose was from Sigma, protein G and protein A conjugated with horseradish peroxidase were from Bio-Rad. CCL13 cells were obtained from The American Type Culture Collection.

2.1. Immunoprecipitation of AMPK α1 and α2 from rat liver

Partially purified AMPK was incubated with excess α1 or α2 affinity purified antibody for 1 h at 4°C. 50 μl of protein G-Sepharose slurry in buffer A (50%, v/v) was added and the mixture incubated for a further 1 h at 4°C. After centrifugation the supernatant was removed and an aliquot used to determine AMPK activity. The supernatant was subjected to a further immunoprecipitation using either the same antibody or an antibody to the other α isoform. The immune complexes were washed extensively with buffer and then analysed for AMPK activity and by Western blotting.

2.2. Western blots

Samples were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membrane. The membrane was blocked by incubation in 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5% Tween 20, 5% low fat milk powder for 1 h at room temperature. The primary antibody was applied in the same buffer and the blot incubated for between 1–12 h at 4°C. After extensive washing with 10 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 0.5% Tween 20, the blots were incubated for 1 h at room temperature with either protein A conjugated with horseradish peroxidase (for primary antibodies raised in rabbits) or protein G conjugated with horseradish peroxidase (for sheep antibodies). After further extensive washing the blots were developed using enhanced chemiluminescence (Boehringer Mannheim).

2.3. Expression of AMPK subunits in CCL13 cells

cDNAs encoding each AMPK subunit were constructed in pcDNA3 (Invitrogen). CCL13 cells were transfected with plasmid DNA (10 μg of each plasmid) by calcium phosphate precipitation [17]. The precipitate was incubated with the cells overnight, followed by a 2 min incubation with phosphate-buffered saline containing 10% (v/v) dimethylsulphoxide. Cells were harvested 48 h post-transfection and lysed by resuspension in 50 mM Tris-HCl, 50 mM NaF, 5 mM

PII S0014-5793(96)01209-4

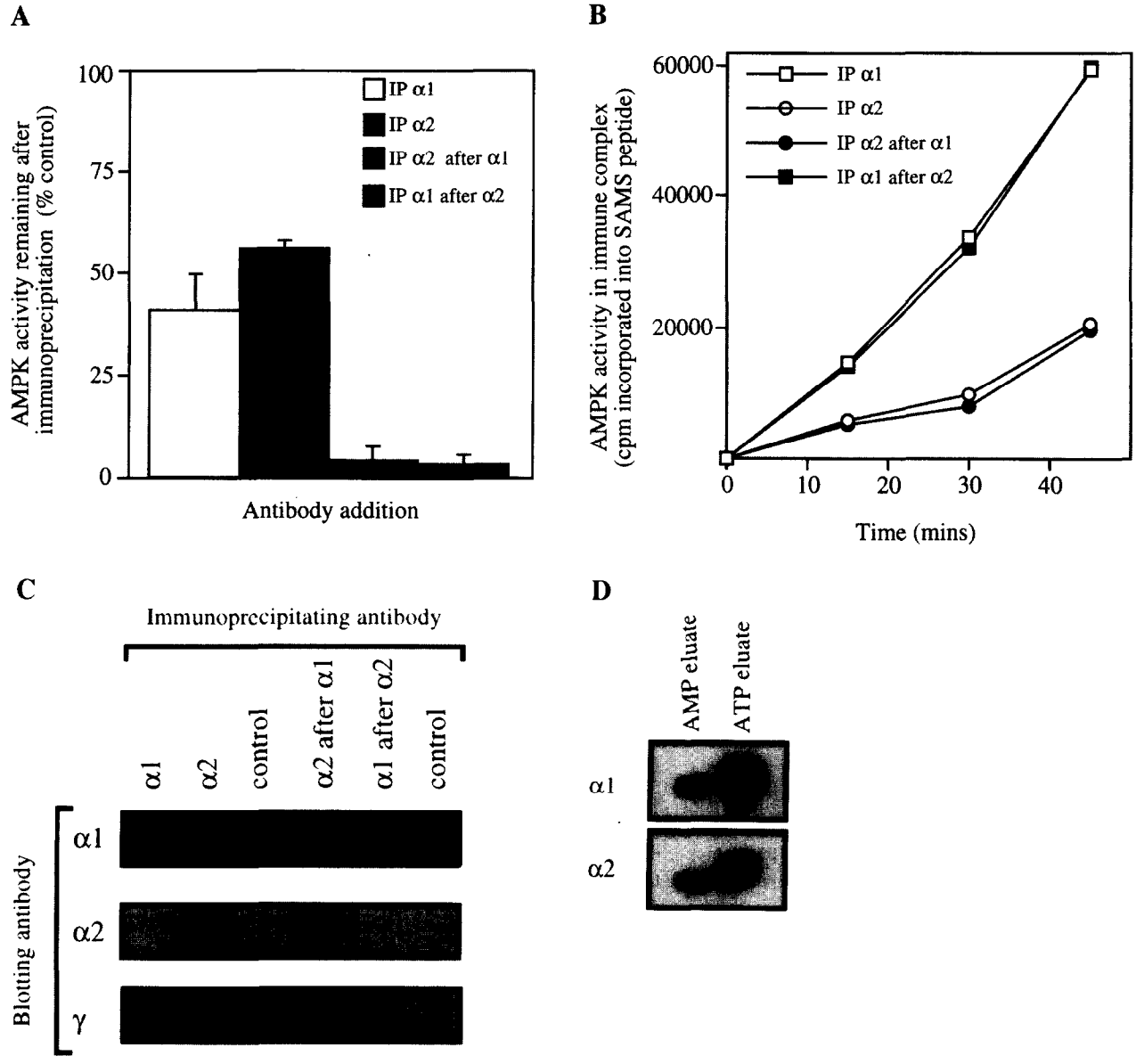


Fig. 1. AMPK, purified up to the gel filtration step, was immunoprecipitated using an excess of either α1 or α2 specific antibodies bound to protein G-Sepharose. A further addition of α1 antibody was added to the supernatant from the α2 immunoprecipitation and vice versa. Sheep anti-rabbit IgG bound to protein G-Sepharose was used in control immunoprecipitations. A: AMPK activity remaining in the supernatant after each precipitation, presented as a percentage of the activity remaining in the control. The results shown are the means of four independent experiments ± S.E.M. B: AMPK activity present in the immune complex from one of the above experiments is shown. A Western blot of each immune complex following SDS-PAGE analysis, probed with either α1, α2 or γ antibodies is shown in C. D: Western blot of the AMP and ATP eluates of AMPK following purification by chromatography on ATP-γ-Sepharose, probed with either α1 or α2 antibodies.

sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 10% (v/v) glycerol, 1% Triton-X100 (buffer A). Insoluble material was removed by centrifugation and the supernatant made to 10% (w/v) with polyethylene glycol. After 10 min on ice the precipitate was collected by centrifugation and resuspended in buffer A.

2.4. AMPK assay

AMPK activity was measured by phosphorylation of the SAMS peptide as previously described [18]. Activities in the immune complexes were measured by addition of the assay reagents directly to the immunoprecipitate. Just prior to removal of an aliquot (20 μl) from the reaction the mixture was centrifuged to pellet the immune complex. Assays to determine peptide specificity were performed at a single concentration of peptide (40 μM) as described elsewhere [14].

3. Results

3.1. Measurement of AMPK α1 and α2 activities in rat liver

Antibodies specific to either α1 or α2, or sheep anti-rabbit IgG as a control, were used to immunoprecipitate AMPK from a partially purified preparation from rat liver. The amount of antibody used had been previously determined to be in excess. Activity remaining in the supernatant after precipitation with anti-α1 antibody was 30–50% of the activity in the control (Fig. 1A). Alternatively, 52–57% of activity remained in the supernatant after immunoprecipitation with anti-α2 antibody. A second immunoprecipitation using the same antibody did not result in any further decrease in activity in the supernatant (data not shown). Conversely, when the

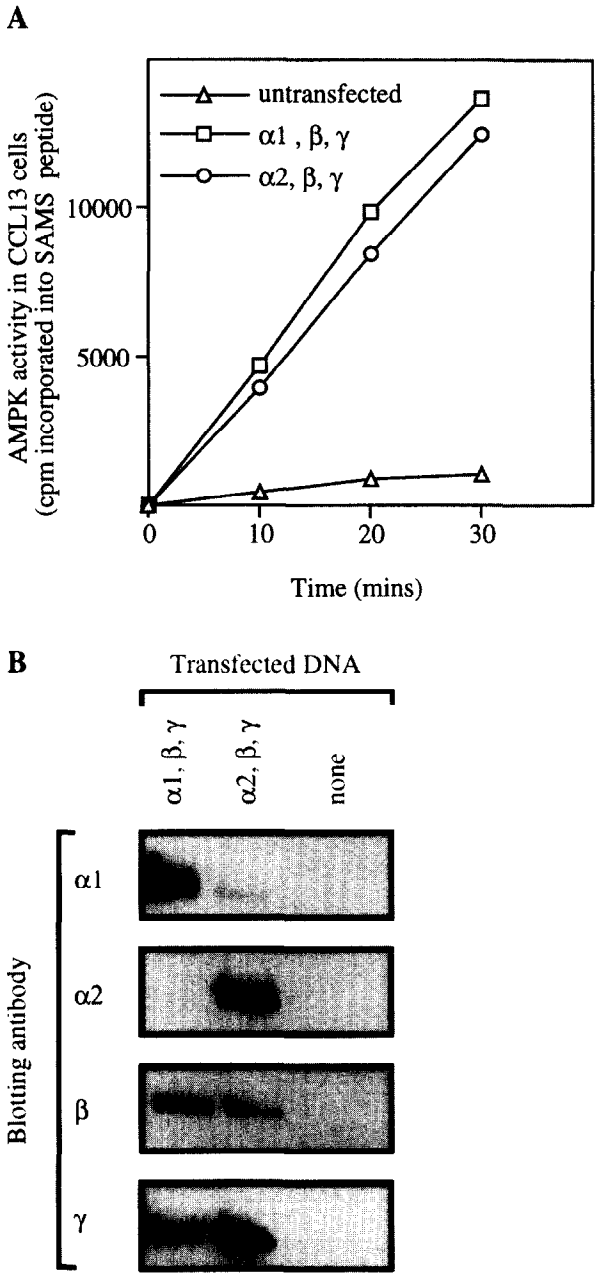


Fig. 2. AMPK activity in a partially purified fraction of CCL13 cells following transient transfection with cDNAs encoding α1, β and γ or α2, β and γ is shown in A. In B, a Western blot of the AMPK subunits, after separation by SDS-PAGE, is shown.

supernatant from the α1 precipitation was followed by immunoprecipitation using α2 antibodies the activity remaining in the supernatant was reduced to 0–5% of the activity in the control. Similarly, when α2 precipitation was followed by immunoprecipitation using α1 antibodies the activity remaining in the supernatant was reduced to 0–6%. AMPK activity determined in the immune complex is shown in Fig. 1B. The activity in the immunoprecipitate due to each isoform specific antibody is the same whether it is measured preceding or following precipitation with the alternative antibody. The activity measured in the immune complex following precipitation using α2 antibody appeared to be significantly less than that using α1 antibody. The most likely explanation for this is that binding of the α2 antibody interferes with kinase activity. To ensure that each antibody was specific for the isoform to which it was raised immunoblots of the immune complexes were probed with both α1 and α2 antibodies (Fig. 1C). The results show that only α1, and not α2, is precipitated by anti-α1 antibody and that α2, and not α1, is precipitated by anti-α2 antibody. These results also demonstrate that α1 and α2 do not associate into a heterodimer. The blots were also probed with anti-γ antibody which showed that a similar amount of γ is associated with either α1 or α2. Neither α1, α2 or γ were precipitated by the control antibody and no activity could be detected in the control immune complex.

The relative contribution of each α isoform to the total AMPK activity remains similar throughout different stages of purification (data not shown). Furthermore, α1 and α2 co-purify throughout purification up to and including chromatography on ATP-γ-Sepharose. Fig. 1D shows a Western blot of the AMP and ATP eluates following chromatography on ATP-γ-Sepharose probed with either α1 or α2 antibody.

3.2. Expression of AMPK in CCL13 cells

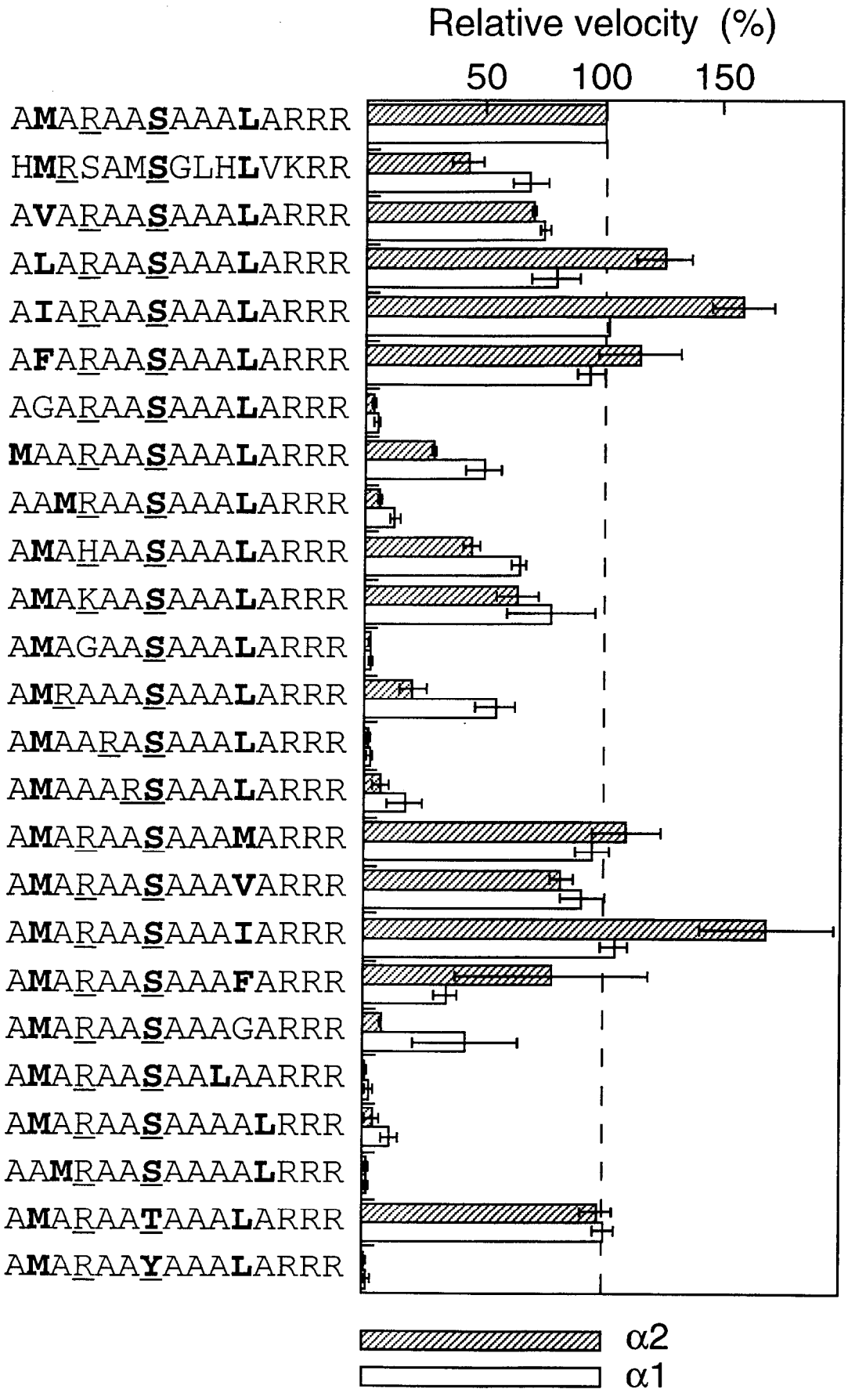
cDNAs encoding α1, α2, β or γ were constructed in a mammalian expression vector under the control of the human cytomegalovirus promoter. α1 or α2 along with β and γ were co-transfected into CCL13 cells. AMPK activity in a partially purified preparation of either untransfected or transiently transfected CCL13 cells was measured 48 h post-transfection and is shown in Fig. 2A. AMPK activity in transfected cells was at least 10-fold higher than the endogenous activity measured in untransfected cells. Co-transfection of either α1 or α2 with β and γ resulted in expression of virtually indistinguishable activities. Fig. 2B shows a Western blot of the partially purified extracts probed with subunit-specific antibodies. Endogenous AMPK in untransfected cells was below the level of detection, whereas all three subunits were readily detectable in transfected cells. Co-transfection of all three subunits was necessary to detect expression of either α, β or γ protein. Similarly, no significant increase in AMPK activity was observed when subunits were expressed either in isolation or in pair-wise combinations (data not shown).

3.3. Substrate specificity of α1 and α2

In order to demonstrate whether α1 and α2 recognise different sequence motifs for phosphorylation, we used a panel of peptides which have been used previously to study the specificity of AMPK and related protein kinases [14]. Fig. 3 shows the relative velocities measured using α1 or α2, prepared by specific immunodepletion of a purified preparation from rat liver which contained both isoforms. There were small differences in specificity between the two isoforms, particularly with respect to the preference for hydrophobic side chain at the P−5 and P+4 position. Similar differences were obtained by assaying α1 or α2 in the immunoprecipitates, or by assaying recombinant α1/βγ or α2/βγ expressed in CCL13 cells (not shown).

4. Discussion

We have shown that AMPK α1 and α2 co-purify throughout purification and that the relative contribution of each isoform to total activity in rat liver is approximately equal. This is in contrast to the results of a recent paper [13] report-


Relative velocity (%)
50
100
150
AMARAASAAALARRR
HMRSAMSGLHLVKRR
AVARAASAAALARRR
ALARAASAAALARRR
AIARAASAAALARRR
AFARAASAAALARRR
AGARAASAAALARRR
MAARAASAAALARRR
AAMRAASAAALARRR
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α2
α1

Fig. 3. Initial rate of phosphorylation of synthetic peptides, expressed relative to the rate obtained with the parent peptide AMARAASAAALARRR. Results for α2 (hatched bars) were obtained by immunoprecipitating α1 from AMPK purified to the gel filtration step, and vice versa for α1 (open bars). Western blots of the supernatants confirmed that they contained only α1 or α2. Results are the mean ± S.E.M. for three determinations. The phosphorylated residue in the peptide is indicated by underlining and bold type, hydrophobic residues by bold type, and basic residues by underlining.

ing that although both isoforms were present, as judged by Western blotting, the α2 isoform accounted for less than 10%, and the α1 isoform for greater than 90%, of the activity in rat liver. Our results are based on immunoprecipitation of AMPK activity using antibodies specific for either α1 or α2. These results confirm previous data in which we used an antibody raised against an α2 specific peptide which depleted activity by 40% [8]. Although the full details of the purification procedure used by Stapleton et al. have not been presented as yet, we consider that the most likely explanation for the discrepancy between the two studies is that their purification protocol results in loss in activity of the α2 isoform. In addition to the finding that α1 and α2 contribute similarly to AMPK activity in tissue extracts, nearly identical activities were detected in cells expressing recombinant AMPK, regardless of which α isoform was used for transfection. It is noteworthy that co-transfection of all three subunits is required in order to detect either expressed protein or activity. When only one or two subunits were transfected neither expressed protein nor activity could be detected. This suggests that either the mRNAs or, more likely the polypeptides, are unstable unless all three subunits are co-expressed in this system. Immunoprecipitation of AMPK using α1 antibodies does not co-precipitate α2 and vice versa (Fig. 1C), indicating that α1 and α2 are not associated into a heterodimer form.

Both α1 and α2 have been shown to bind β and γ in a 1:1:1 ratio [13,15]. Western blotting of the immunoprecipitates from rat liver indicate that very similar amounts of γ co-precipitate with each α isoform. This suggests that the expression of α1- and α2-containing complexes in rat liver is similar. Since α1 and α2 contribute roughly equally to AMPK activity this suggests that both isoforms have similar specific activities. The amino acid sequences of α1 and α2 are 77% identical [13]. The major regulatory phosphorylation site within the α subunit has been mapped to threonine-172 [7] and the sequence surrounding this site is identical in both isoforms. In addition both isoforms are stimulated to the same extent by AMP, and are both widely expressed in rat tissues [13]. These similarities raise the question as to whether there is any difference in the role of the isoforms within the cell. One possible functional difference between the two is their substrate specificity. In order to address this possibility we investigated the phosphorylation of a panel of synthetic peptides, which had been used previously to determine the phosphorylation recognition motif of what we now know to have been a mixture of α1 and α2 [14]. The results for each isoform were qualitatively similar to those obtained previously with the mixture. Both isoforms recognise the φ(X,β)XXS/TXXXφ motif (where φ represents a hydrophobic residue and β a basic residue) but there are quantitative differences with respect to preference for the hydrophobic residues at the P−5 and P+4 positions. Relative to α1, α2 appears to prefer leucine and isoleucine over methionine at the P−5 position and it also appears to prefer isoleucine over leucine at P+4. α1 also seems to be more tolerant of the basic residue being at the P−4 rather than P−3 position. These differences, although subtle, indicate that within the cell α1 and α2 could phosphorylate different substrates, at different rates, allowing more flexibility in the phosphorylation of downstream targets within the AMPK cascade. Further studies are necessary to elucidate the exact roles of both isoforms in vivo.

Acknowledgements: We would like to thank Steve Davies for supplying partially purified AMPK. This work was funded partly by the Medical Research Council, London. A.W. was supported by an Intermediate Research Fellowship from the British Heart Foundation, I.S. by a BBSRC studentship and D.G.H. by a Programme Grant from the Wellcome Trust.

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