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AMP-activated protein kinase isoenzyme family: subunit structure and chromosomal location

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Abstract The AMP-activated protein kinase (AMPK) consists of catalytic α and non-catalytic, β and γ (38 kDa) subunits and is responsible for acting as a metabolic sensor for AMP levels. There are multiple genes for each subunit and we find that rat liver AMPK-α2 isoform catalytic subunit is associated with β1 and γ1 and not with β2 or γ2 subunit isoforms. The β1 and γ1 isoforms are also subunits of the α1 isoform. The sequence of cloned human AMPK-β1 is 95% identical in amino acid sequence with rat β1. Human chromosomal localizations were determined for AMPK-α1 (5p11-p14), AMPK-β1 (12q24.1-24.3) and AMPK-γ1 (12q12-q14), respectively.

Key words: AMP-activated protein kinase; Isoform; Gene; Chromosome

1. Introduction

The AMP-activated protein kinase (AMPK) consists of an α, catalytic subunit (63 kDa) and non-catalytic, β (40 kDa) and γ (38 kDa) subunits [1–3]. Co-expression of the non-catalytic β and γ subunits is required for optimal activity of the α catalytic subunit [4]. The AMPK phosphorylates a number of key enzymes involved in the control of lipid metabolism: acetyl-CoA carboxylase, HMG-CoA reductase and hormone-sensitive lipase [5]. It is activated by elevation of intracellular 5′-AMP caused by arsenite and heat shock and is thought to function primarily in stress responses [6]. Studies on HMG-CoA reductase regulation have reinforced the concept that the AMPK plays a role in metabolic stress responses, since mutation of Ser-871, the AMPK phosphorylation site in HMG-CoA reductase, to Ala, blocked phosphorylation by the AMPK and reduction in HMG-CoA reductase activity caused by ATP depletion, but did not affect the transcriptional control of HMG-CoA reductase [7]. The activation of the AMPK by AMP results from three contributing mechanisms: direct allosteric activation of the enzyme, AMP activation of an upstream kinase [8] and AMP inhibition of AMPK dephosphorylation [9].

Recently, we found that multiple isoforms of the AMPK are present in liver [10]. Both α1 and α2 isoforms could be stimulated by 5′-AMP and contained non-catalytic β and γ subunits. The two isoforms of the AMPK, α1 and α2, are 90% identical in the catalytic core region, but have divergent COOH-terminal tails (60% identical). In addition, EST sequence analysis of the β and γ non-catalytic subunits has revealed the presence of isoforms specific for each subunit within the human genome [11]. Since the AMPK consists of a family of isoenzymes, it was important to establish which β and γ subunit isoforms are bound to each catalytic isoform. Rat and porcine liver AMPK-α1 subunits are associated with β1 and γ1 [3,12] but the identity of the non-catalytic subunits associated with AMPK-α2 was uncertain and it seemed reasonable that it may associate with a distinct set of β and γ non-catalytic subunits. In the present study, we have purified the rat liver AMPK-α2 isoform and show by protein sequencing that it is associated with β1 and γ1 subunits. The chromosomal localization of the corresponding human genes has been determined.

2. Materials and methods

2.1. AMPK-α2 purification

Peptide antibodies were raised against AMPK-α2 (352-366) and (490-514) as previously described [10]. Affinity purified antibodies (400 μg each/ml beads) were coupled to 4 ml of Tosyl-activated Dynabeads (DYNAL, Oslo, Norway) according to the manufacturer's instructions. Extracts of rat liver were processed as described previously for the purification of AMPK-α2 [13]. The fraction containing the AMPK-α2, which is not retained by the substrate affinity column [13] was incubated overnight at 4°C with the magnetic beads were then collected into one tube, washed with 5×1 ml of phosphate-buffered saline and 5×1 ml of 50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.5 M NaCl and 1% Triton X-100 (w/v). The antigen was eluted with 4×1 ml of 0.2 M glycine, pH 2.0, precipitated with trichloroacetic acid (6%) and analysed by SDS-PAGE.

2.2. Protein sequencing

Peptides were derived from rat β and γ subunits of AMPK separated by SDS-PAGE using in-situ proteolysis described previously [1]. Briefly, Coomassie Blue-stained gel slices were excised and placed in the funnel compartment of a Hewlett Packard G1004B Chemstation where they were washed extensively in water, reduced in 5 funnel volumes (≈5 ml) of 0.2 M Tris-HCl buffer containing 10 mM DTT and 1 mM EDTA, pH 8.5, at 45°C (3 h) and alkylated with 5 funnel volumes of 1% 4-vinylpyridine in 0.2 M Tris-HCl buffer, 1 mM EDTA, pH 8.5, at ambient temperature (2 h). The gel slices were destained in 7 funnel volumes of 50 mM ammonium bicarbonate, 50% acetonitrile at 65°C for 3 h and dried in a centrifugal freeze drier. The gel slices were rehydrated in digestion buffer containing protease (30 μl/slice from a single lane of a 1 mm gel). Modified Trypsin (Promega) or achrombacter endoproteinase Lys-C (Wako) in either 10% acetonitrile (v/v) 50 mM ammonium bicarbonate buffer for tryp-

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tic digests or adjusted to pH 9.2 with ammonia solution for Lys-C digests. The protease to substrate ratio was approximately 1:20 (w/w). The rehydrated gel slices were covered with an additional 200 μl of digestion buffer and incubated at 37°C overnight. Excess digestion buffer was removed and reserved. The gel slices were extracted with 200 μl of 2% (v/v) trifluoroacetic acid (TFA) and incubated in a sonicating water bath for 1 h. The supernatant was removed and pooled with the reserved digestion buffer. This procedure was repeated with 200 μl of 40% acetonitrile 0.1% TFA and finally 200 μl of 80% acetonitrile 0.1% TFA and the combined gel eluates were dried in a centrifugal freeze drier. The digest mixture was reconstituted in 6 μl of 100% TFA, vortexed then 300 μl of 6 M guanidine hydrochloride was added and the mixture was subject to chromatography on Nucleosil C18 5 μm 300 Å reversed-phase glass-lined column (SGE) (1×250 mm) on a Pharmacia SMART system using a linear 0-80% acetonitrile 0.1% TFA gradient over 120 min at 40 μl/min and the 214 nm peaks collected automatically. Peak purity was assessed using a Perspective Biosystems Voyager DE MALDI time-of-flight mass spectrometry with samples crystallised in the presence of α-cyano-4-hydroxycinnamic acid and sequenced on a Hewlett Packard G1000A Protein Sequencer utilising Routine 3.5 Edman degradation chemistry as recommended by the manufacturer.

2.3. Isolation of human AMPK cDNAs and genomic clones

A genomic AMPK-α1 clone was obtained by screening a human PI genomic library (Genome Systems, Inc.) with a 387 bp region of AMPK-α1 cDNA (681-1065) and subcloned into two smaller fragments. Following sequencing with AMPK-specific oligonucleotides, a 5000 bp subclone corresponding to the non-catalytic region of AMPK-α1 was chosen as the probe for chromosome localization.

Human AMPK-β1 was identified as an EST (R20494) following BLAST algorithm [14] searches of the genome database with rat liver AMPK-β1. R20494 was obtained from IMAGE clones and its cDNA sequence determined by dideoxy chain termination using vector and AMPK-β1-specific oligonucleotides. Comparison of human AMPK-β1 with rat AMPK-β1 [11] revealed 95% and 89% identity at the amino acid and nucleotide levels, respectively. Human AMPK-γ1 was isolated as previously described [11].

2.4. Chromosomal localization

The following probes were employed: AMPK-β1 (1.75 kb cDNA in vector, R20494), AMPK-α1 (~5 kb genomic DNA in vector), and AMPK-γ1 (1.6 kb cDNA in vector, human fetal liver). The probes were nick-translated with biotin-14-dATP and hybridized individually in situ at a final concentration of 10-20 ng/ml to normal male metaphases. The fluorescence in situ hybridization (FISH) method was modified from that previously described [15] in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification). Images of AMPK-β1 preparations were captured by a cooled CCD camera using the Cyto Vision Ultra image collection and enhancement system (Applied Imaging Int. Ltd.). FISH signals and the DAPI banding pattern were merged for figure preparation.

3. Results and discussion

3.1. Subunit structure of the AMPK-α2 isoform

The AMPK-α2 isoform was purified from rat liver by immunoaffinity chromatography using antibody against the AMPK-α2 catalytic subunit (Fig. 1). The identity of the AMPK-α2 catalytic subunit was verified by peptide sequence analysis with 17% of the subunit sequence obtained by in situ proteolysis. This included two peptides derived from the catalytic core and three peptides from the COOH-terminal domain (Fig. 2A). The sequence comparisons confirm that it was the α2-isoform of the AMPK catalytic subunit which was immunoaffinity purified. The peptide sequences obtained for the corresponding β subunit accounted for 46% of the expected 270 residues of protein sequence and were identical to the β1 sequence previously identified as the β subunit isoform associated with the α1 catalytic subunit (Fig. 2B). The

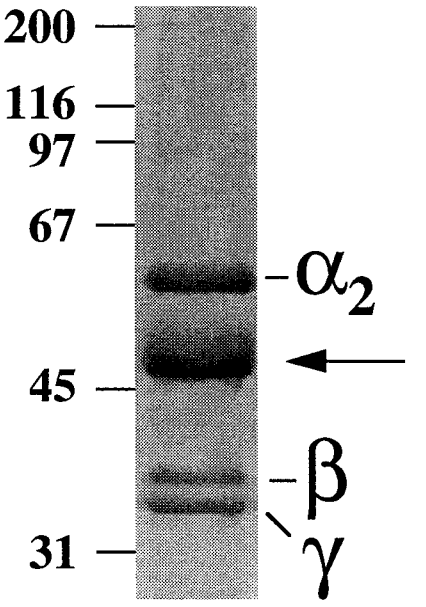

Fig. 1. Subunit structure of rat liver AMPK-α2. AMPK-α2 was purified as described in Section 2. Shown is an SDS-PAGE (13%) containing the AMPK-α2 catalytic subunit together with the AMPK non-catalytic subunits, β and γ. The arrow indicates the presence of the antibody heavy chain. Molecular mass standards are shown in kDa.

peptide sequence derived from the γ subunit associated with the AMPK-α2 isoform accounted for 43% of the sequence and this was identical to the γ1 isoform (Fig. 2C). These results demonstrate that the liver AMPK-α2 catalytic subunit associates with the same set of non-catalytic subunits as the AMPK-α1 catalytic subunit and not with other isoforms of the non-catalytic subunits. Previously we found that both β1 and γ1 could associate with either α1 or α2 when all three subunits were transfected into COS cells [4].

Recent genome sequencing has revealed multiple isoforms of both the β and γ subunits with two isoforms of the γ subunit revealed by EST sequences (Fig. 2C). Evidence for a γ2 isoform is based on partial-length cDNAs from human brain, accession numbers H15390 and H06773, and human liver, accession number H64260 (Fig. 2C) We raised anti-peptide antibodies to the putative γ2 isoform (cDNA derived sequence, LTPAGAKQKETETE). Immunoprecipitation of the post Blue-Sepharose fractions [13] of the rat liver AMPK preparation with the anti-γ2 antibody indicated that it was associated with α- and β-like subunits but the quantity of material obtained was insufficient to allow amino acid sequence analysis.

3.2. Chromosomal localization

Twenty metaphases from a normal male were examined for fluorescent signal using the AMPK-α1 probe. Nineteen of these metaphases showed signal on one or both chromatids of chromosome 5 in the region 5p11-p14; 85% of this signal was at 5p12. There were a total of 39 non-specific background dots observed in these 19 metaphases. A similar result was obtained from hybridization of the probes to 15 metaphases from a second normal male (data not shown). To confirm that the signal was on chromosome 5 the Oncor probe D4Z1 was

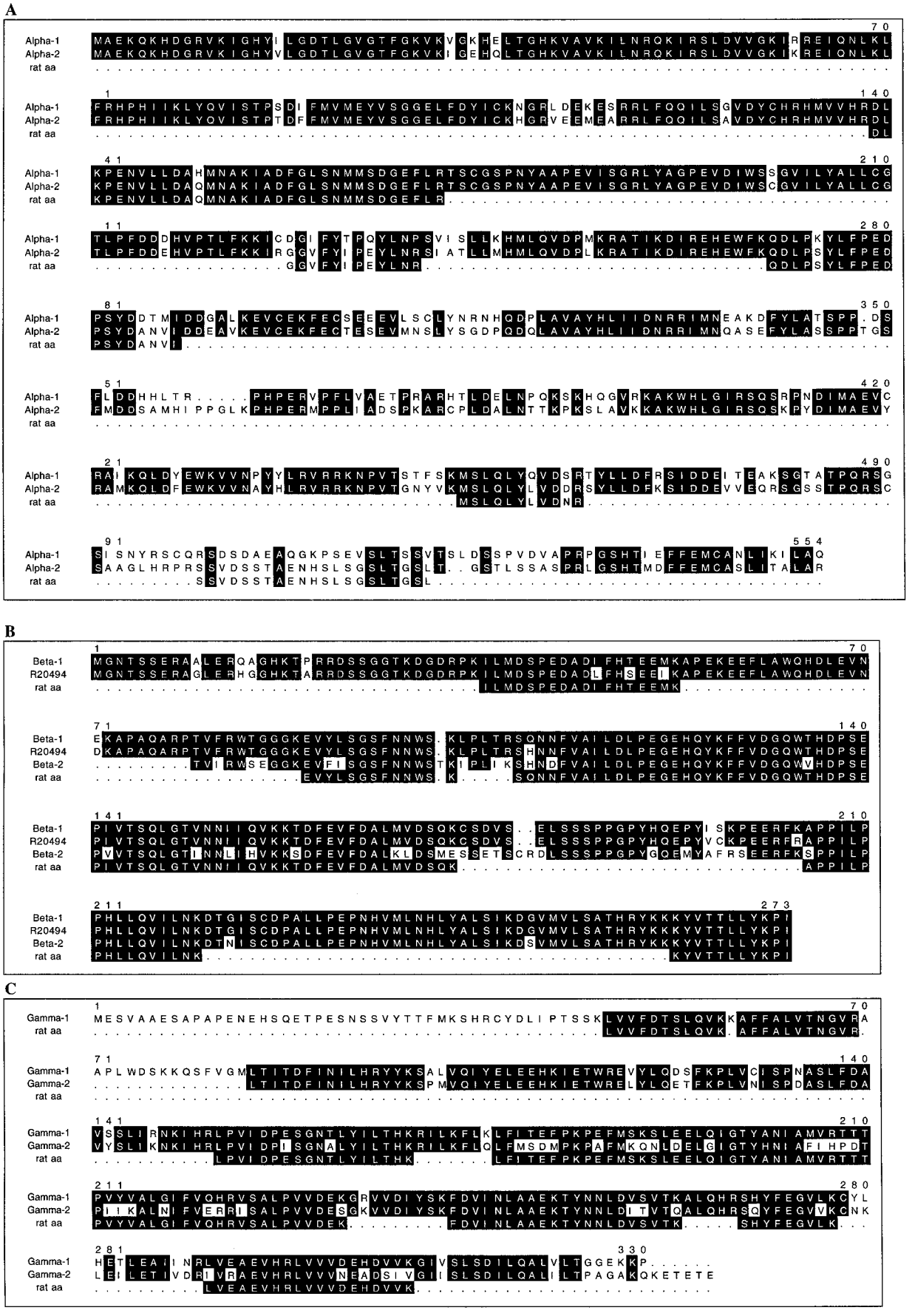
A
Alpha-1
Alpha-2
rat aa
B
Beta-1
R20494
Beta-2
rat aa
C
Gamma-1
Gamma-2
rat aa

Fig. 2. AMPK-α2 isoform subunit sequences. AMPK-α2 was purified and subjected to partial amino acid sequencing as described in Section 2. The sequences shown in panels A–C were aligned with the Pileup program (GCG, University of Wisconsin [18]) and formatted, with the residues identical to the AMPK isoenzyme-1 isoform being shaded. A: Alignment of the catalytic isoforms, AMPK-α1 (Alpha-1) and AMPK-α2 (Alpha-2) and the peptide sequence derived from the purified AMPK-α2 catalytic subunit shown in Fig. 1. B: Alignment of rat liver AMPK-β1 [11], human brain AMPK-β1 (R20494), mouse AMPK-β2 isoform (partial-length cDNA from mouse fetal lung, accession number W07176, as identified in the Genebank by BLAST searching) and the peptide sequence derived from the purified AMPK-β1 non-catalytic subunit shown in Fig. 1. C: Alignment of rat liver AMPK-γ1 [11], human AMPK-γ2 isoform (partial-length cDNAs from human brain, accession numbers H15390 and H06773, and human liver, accession number H64260, as identified in the Genebank by BLAST searching) and the peptide sequence derived from the purified AMPK-γ1 non-catalytic subunit shown in Fig. 1.
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hybridized simultaneously. D4Z1 hybridizes to the centromere region of chromosome 4, so chromosome 4 could be separated from chromosome 5, hence allowing unequivocal identification of the B group chromosomes. In contrast the AMPK-α2 gene mapped to 1p31 (OMIM 600497) [16].

Twenty-five metaphases from a normal male were examined for fluorescent signal with the AMPK-β1 probe. Twenty-three of these metaphases showed signal on one or both chromatids of chromosome 12 in the region 12q24.1-q24.3; 90% of this signal was at 12q24.1 (Fig. 3). There was a total of seven non-specific background dots observed in these 25 metaphases. A similar result was obtained from hybridization of the probe to 21 metaphases from a second male (data not shown). The *AMPK-β2* (Fig. 2B) gene has not yet had its chromosomal location determined.

Twenty-five metaphases from a normal male were examined for fluorescent signal using the AMPK-γ1 probe. All of these metaphases showed signal on one or both chromatids of chromosome 12 in the region 12q12-q14; 77% of this signal was at 12q13.1. There was a total of 20 non-specific background dots observed in these 25 metaphases. A similar result was obtained from hybridization of the probes to 20 metaphases from the second normal male (data not shown). Twenty metaphases expressing the rare folate-sensitive site FRA12A, and showing signal, were then examined. Of these, 17 showed signal proximal to the fragile site, two showed signal central and one showed signal distal. We therefore infer that AMPK-γ is proximal to FRA12A. The precise location of FRA12A was described by Sutherland and Hecht as in the middle of band 12q13.1 [17]. The *AMPK-γ2* gene (Fig. 2C) has recently been mapped to chromosome 7 by Genethon, marker A005S22 (D7S676-D7S505) corresponding to region 7q35-36.

The results reported here show that the genes for the AMPK subunits (α, β, γ) and their isoforms are distributed over a number of chromosomes. In liver the AMPK-α2 catalytic subunit associates with β1 and γ1 non-catalytic subunits which also associate with AMPK-α1. Thus the marked divergence in the COOH-terminal sequences between the AMPK-α2 and AMPK-α1 catalytic subunits is not responsible for associating with different non-catalytic subunits.

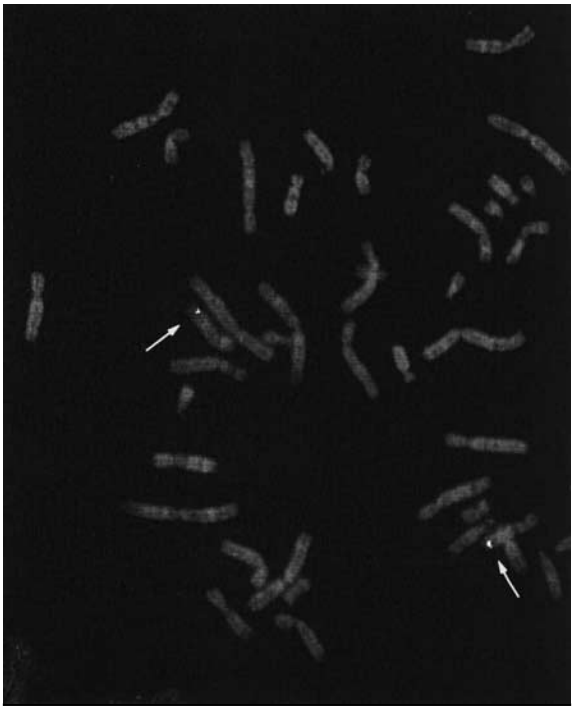

Fig. 3. Metaphase showing FISH with the AMPK-β1 probe. Normal male chromosomes stained with DAPI. Hybridization sites on chromosome 12 are indicated by arrows.

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